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Self-healing and Adhesive Artificial Tissue Implant for Voice Recovery

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바이오엔지니어링전공

이승훈

ABSTRACT

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Seunghun S. Lee

Interdisciplinary Program of Bioengineering

The Graduate School

Seoul National University

Loss of voice after vocal fold resection due to laryngeal cancer is a significant problem resulting in a low quality of life. Although there were many attempts to achieve functional restoration of voice, challenges to regenerate vocal fold still remains due to its unique tissue mechanical characteristics such as pliability that produces phonation via vibration. In this study, we developed mechanically compliant interpenetrating polymer network (IPN) hydrogel based on polyacrylamide (PAAM) and gelatin that matches physical and functional properties with native vocal fold tissue. The mechanical properties of this PAAM/gelatin (PG) hydrogel were modulated and optimized for vocal fold engineering by adjusting PAAM/gelatin ratio. In addition, PG hydrogel showed minimal foreign body

reaction upon implantation, and the hydrogel displayed strong resistance to dehydration condition. Furthermore, PG hydrogel demonstrated self-healing ability that may allow ad-hoc implant augmentation. In addition, tough adhesion of PG hydrogel resulted in stable attachment to vocal fold tissues. Finally, we demonstrated the functional restoration of voice on *ex vivo* canine model by implanting PG hydrogel as an artificial vocal fold tissue.

Keywords: biomedical applications, biomimetic, hydrogels, artificial vocal fold, phonation, polyacrylamide, gelatin, tissue engineering,

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1. Introduction

Vocal fold, located within larynx at the top of the trachea, is a complex and multilayered structure that is responsible for phonation via vibration.[1-4] Its delicate and soft tissue is easily impaired by accident, environmental factors or diseases which results in vocal disorders.[5, 6] Furthermore, up to 9% of the populations have voice abnormalities and 29% of the populations experience a voice disorder during their life. [5, 7-9] In particular, voice loss after laryngectomy due to laryngeal cancer is a crucial problem resulting in low life quality. Although there were numerous studies that attempt to achieve functional restoration of voice, the optimal solution for voice recovery has not yet been discovered. It is due to its unique mechanical characteristics such as pliability that produces phonation via vibration.[6] For instance, Teflon, silicon and bovine collagen were used as permanent materials for vocal fold augmentation but with little success in voice recovery.[10] In another study, Pitman et al. transplanted temporalis fascia for vocal fold injury, but the result did not show significant improvement in voice recovery.[11] There were studies involving hydrogels, via implantation, however, clinical application of hydrogel such as thiol-modified hyaluronic acid was not suitable due to long gelation time required to form stable hydrogels, tissue adhesion, stretchability, short duration of action and practical feasibility for phonation.[12-21] Additionally, most biomaterials mentioned for vocal fold repair was approached by augmentation via injection.[1, 12, 14, 17, 22] This means it would be not proper for injection when there is

insufficient volume to inject due to too much resection or severe harden tissue due to scar change.

In this study, we present PAAM/gelatin (PG) based interpenetrating polymer network (IPN) hydrogel with self-healing ability and strong tissue adhesion that can be applied as an artificial vocal fold implant. IPN hydrogel, formed by combining two different pre-polymer solutions, may surmount downside of the individual polymer by changing its properties such as mechanical strength.[23] Thus, one advantage of PG hydrogel is the simple modulation to function as vocal fold tissue from the physical entanglement of two materials with distinct characteristics, PAAM and gelatin. PAAM has been studied by researchers for a long time due to its biocompatibility and hydrophilicity which make it promising materials for biomedical applications such as soft contact lenses.[24-27] Gelatin is temperature dependant natural, biocompatible and biodegradable polymer derived from collagen that forms triple helices.[28-30] Its temperature dependent denaturation characteristic serves as self-healing property.[31-33] However, gelatin hydrogel alone cannot function as a vocal fold due to its weak and unmatching mechanical property.[34, 35] We hypothesize that IPN hydrogel formed by PAAM and gelatin may provide stretchable, tough tissue adhesive and self-healing characteristics that may suitable for vocal fold tissue augmentation.[13, 23, 36] In order to utilize PG hydrogel as an artificial vocal fold, we explored mechanical and rheological properties of PG hydrogels with different ratios of PAAM to gelatin. Then, its

biocompatible properties such as cell cytotoxicity, dehydration resistance, and swelling ratio were analyzed to see its durability and safety for implantation. In our study, mechanisms of tissue adhesion via anchoring effect of gelatin from PG hydrogel was observed. In addition, we report that tissue adhesion of PG hydrogel was enhanced by interfacial silica microparticles. Finally, in ex vivo canine vocal fold model, PG hydrogel resulted in full voice recovery.

2. Experimental section

2.1 Materials

Acrylamide (AAm), Gelatin from bovine skin (type B), N,N'-Methylenebisacrylamide (MBAA), Ammonium persulfate (APS) and N,N,N',N'-Tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich.

2.2 Synthesis of PG Hydrogel

2.2 M of AAm solution was prepared by dissolving 4.69 g of AAm in 30 ml of distilled water. 2.2M AAm solution was mixed with 7.035 mg of MBAA. 100 mg/ml of APS was prepared by dissolving in distilled water. 200 mg of gelatin was prepared with 2 ml of distilled water and stored in 60°C chambers to make 10% (w/v). Table 1 shows four groups of PG hydrogel with different ratio of PAAM to gelatin (PAAM only group, 19:1 group, 9:1 group and 7:1 group) that were used in this experiment. After mixing AAm solution and gelatin solution gently to make the solution homogenous, 150 µl of APS solution and 6 µl of TEMED were added to the mixed solution and the mixture was gelled at room temperature overnight.

2.3 Mechanical Testing

For compression test, the PG hydrogels were synthesized with cylindrical shape (diameter: 11 mm, height: 10 mm). Compression mechanical tests were performed

by using a universal-testing-machine (Shimadzu EZ-SX, Japan) with the loading rate of 3 mm/min. For tensile test, the PG hydrogels were synthesized with a rectangular shape (length: 36 mm, width: 17 mm, height: 2 mm). For the mechanical testing machine to grasp the end of the hydrogel, grasping part of front and backside of the PG hydrogel was boned to a rigid polyethylene terephthalate (PET) film with super glue. Tensile mechanical tests were performed by using a universal-testing-machine (Instron 5966, Instron Corporation) with the loading rate of 30 mm/min.

2.4 Rheological testing

Rheological properties of hydrogels were tested using rheometer (MCR 302, Anton-Paar, Austria). The PG hydrogels were synthesized with cylindrical shape (diameter: 25 mm, thickness: 2 mm). Amplitude sweep test was applied from 0.01 % to 100 % of shear strain at the controlled angular frequency of 10 rad/s to determine the rheological change related to the shear strain. The frequency sweep testing measurement was carried out from 1 Hz to 100 Hz at a shear-strain of 3 % to analyze the change of storage and loss modulus related to the oscillatory frequency. Thixotropy-loop test was performed by alternating 3% and 80% of shear strain for every 20 seconds at a constant frequency of 1Hz to investigate the shape recovery behavior of PG hydrogel.

2.5 Swelling and reswelling test

Hydrogels with a diameter of 11 mm and height of 10 mm cylindrical shape were lyophilized to measure the dry weight. Then, the lyophilized hydrogels were submerged in distilled water and measured the weight of swollen state after 0, 1, 2, 4, 6, 12,24 and 48 hours of swelling. After swelling, the hydrogels were lyophilized to remove water to measure the dry weight. Swelling Ratio (Q) was calculated as weight of swollen hydrogel divided by the weight of dried hydrogel times hundred. Then, hydrogels were reswelled with the same method as swelling test to see if there was any hydrogel network deformation during first swelling process that might affect swelling property.

2.6 Dehydration resistance test

The hydrogels were stored in 37°C chamber, and dehydrated weights of hydrogels were measured from 0 day to 20 days. To see the change of dehydration resistance property in different humidity at 20% and 60% of humidity, a beaker filled with distilled water was stationed near the hydrogels in 37°C chamber and the dehydrated weights were measured from 0 day to 20 days. Dehydration resistance was calculated as a percentage of remained hydrogel weight after dehydration divided by initial hydrogel weight before dehydration.

2.7 Scanning Electron Microscopy

Hydrogel samples were lyophilized and coated with platinum sputtering for field emission scanning microscopy (FE-SEM) (FESEM SIGMA, Carl Zeiss, Germany). Images were analyzed with ImageJ software (NIH, United States) to analyze the average pore area of lyophilized hydrogel by converting to 8-bit and adjusting threshold. Region of Interest Manger function was used to measure the multiple-pore areas of the hydrogel. Based on the data of multiple pore areas, the areas were converted to calculate the average pore area.

2.8 Live & Dead assay

First, gel-conditioned media was prepared by incubating PG hydrogels in C2C12 growth medium (Dulbecco's modified Eagle's medium; DMEM, Thermofisher Scientific) which contained 10% fetal bovine serum (10438-026, Gibco), 1% penicillin-streptomycin (15140122, Gibco) and 1% L-glutamine (25030-081, Gibco) for a day in order to see any cytotoxicity released from hydrogel. Adipose derived stem cells (ADSCs) were obtained from Bundang Seoul National University Hospital, Bundang, Korea. The ADSCs were seeded in 24 well and cultured in the gel-conditioned media for 3 days. After 3 days of culture, cells were stained for 10min in 0.5uL/mL of calcein-AM and 2uL/mL of ethidium homodimer-1 (Eth-1) from Live/Dead assay kit (Invitrogen). The cells were visualized with EVOS (AMF4300, Life

Technology) and viability was calculated as the number of live cells per the number of total cells.

2.9 Gelatin release test

Weighed PG hydrogels with different ratio of PAAM/gelatin were immersed in distilled water at room temperature. At selected time points, the distilled water was removed and collected for gelatin release analysis. The released gelatin in a medium was determined by colorimetric protein assay using the BCA method under the manufacturer's protocol (Pierce BCA Protein Assay Kit, ThermoFisher Scientific, USA), with bovine serum albumin (BSA, MP Biomedicals, USA) as a standard. The absorbance of each solution at 750 nm was measured using a UV-Vis spectrophotometer (Infinite 200 PRO TECAN Ltd., Switzerland).

2.10 Self-healing test of PG hydrogel

Two cylindrical shapes of hydrogels were labeled with commercially available watercolor paints, blue and green, by mixing with distilled water before synthesis of the hydrogel. To have congruent shape, 50 ml syringe was used as a mold for hydrogels. After synthesis, two hydrogels were cut into a disk shape and reattached with different colors sequentially. The reattached hydrogel was stored in the 50 ml syringe to prevent askew attachment and to give pressure to reduce the areas that were not contacting between hydrogels. After giving some pressure, the hydrogel

was put in the 60°C chamber for one hour, so the gelatin can move between hydrogels to have self-healing. Then, the hydrogel was put at room temperature to fix the gelatin. Figure 4a shows the graphical demonstration of the self-healing test.

2.11 Synthesis of PG hydrogel with Fluorescein isothiocyanate(FITC)-gelatin

FITC was purchased from Sigma-Aldrich. In order to check how much gelatin was able to penetrate through hydrogel or tissue, FITC-gelatin was synthesized first. 1mg of FITC was dissolved in 0.1mL of DMSO to 10 %(w/v) gelatin solution and the mixture was stored at 60℃ for one hour. The solution was placed in 3.5K MW dialysis tube and dialyzed in distilled water for 5 days to remove unbound FITC. Distilled water was freshly replaced every day for dialysis. On the last day, the FITC-gelatin solution was freeze-dried and used to synthesize the PG hydrogel with FITC-gelatin under the same protocol of PG hydrogel synthesis.

2.12 Self-healing test of PG hydrogel with FITC-gelatin

Two groups of hydrogels were synthesized: PG hydrogel with FITC-gelatin and PG hydrogel without FITC-gelatin. Synthesized PG hydrogel with FITC-gelatin was attached with the PG hydrogel without FITC-gelatin. After attachment, hydrogels were stored in the 60℃ chamber for one hour to have self-healing. Then, hydrogels

were cooled down to room temperature and cut to see the cross-section of contacting area between hydrogels via confocal laser scanning microscopy (CLSM). Intensity by penetration distance from the contacting area was calculated by using Zen lite (Zeiss, Germany) software.

2.13 Adhesion of PG hydrogel with FITC-gelatin to porcine cardiac tissue

A porcine cardiac tissue was obtained from a local store. PG hydrogel with FITC-gelatin was attached to cardiac tissue of porcine. Then, the sample was cut and OCT embedding was performed to see the interface contacting area between the hydrogel and cardiac tissue. Firstly, samples were fixed in 4% paraformaldehyde overnight at 4℃ and then placed in 20%(w/v) sucrose solution overnight. After maintaining a cell and hydrogel structure, samples were cut into a small piece and the interface was placed at the bottom of the cryomolds. Empty space was filled with OCT (Sakura Fineteck) without any bubbles. Then, the cryomolds were frozen on the liquid nitrogen, forming cryoblocks. Samples in cryoblocks were cryosectioned at the 10μm thickness and visualized via confocal laser scanning microscopy (CLSM). Intensity by penetration distance from the contacting area was calculated by using Zen lite (Zeiss, Germany) software.

2.14 Peeling test

A porcine skin was obtained from a local store. Adhesion tests proceeded with 180-degree peeling test. PG hydrogels were cut into a rectangular shape (length: 4 cm, width: 3 cm and height: 0.2 cm) and were attached to the porcine skin with similar size. For hydrogel with silica particle group, 200 µl of silica particle solution (Ludox TM-50 colloidal silica) was applied on the surface of the hydrogel before attachment. Ludox TM-50 colloidal silica was purchased from Sigma-Aldrich. For the mechanical testing machine to grasp the end of hydrogel and porcine skin, front and back side of the PG hydrogel and the porcine skin was boned to a rigid polyethylene terephthalate (PET) film with super glue. A universal testing machine (Shimadzu EZ-SX, Japan) was used to record the force and stroke while the samples were applied with unidirectional tension at the loading rate of 50 mm/min.

2.15 *Ex vivo* adhesion Test of PG Hydrogel for Cardiac Application

A porcine heart was obtained from a local store. A circular defect of 8 mm diameter was created with biopsy punch on the ventricle wall of the heart. Circular PG hydrogel with a diameter of 11 mm was attached to seal the hole of the heart. In the case of PG hydrogel with SP, 100 µl of silica particle solution (Ludox TM-50 colloidal silica) was applied on the surface of the hydrogel before sealing the hole. Ludox TM-50 colloidal silica was purchased from Sigma-Aldrich. The blood was injected via aortic artery to fill the heart with a 50ml syringe. Blood was continuously injected as soon as there was a loss of blood through the hole during the test. Duration

of the test was 180 seconds and blood that was leaked from the hole was collected in the beaker and measured after the test. Photos and videos were recorded to examine leakage at the defect site and to count the number of blood drops every 10 seconds during the test to see how the vessel sealing effect of hydrogel changes over time.

2.16 Recording of Hydrogel Implanted Vocal Fold Vibration with High-Speed Camera and Analysis

Canine vocal fold was resected surgically before recording. For the hydrogel implanted vocal fold sample, 9:1 PG hydrogel was used. 100 µl of silica particle solution (Ludox TM-50 colloidal silica) was applied on the surface of the hydrogel and the hydrogel was attached on the resected vocal fold. To perform functional analysis, vocal fold vibration tests were examined by the method of excised laryngeal setup from Kwon et al.[37, 38] According to literature, the vocal fold vibrations during induced vocal fold phonation were recorded by a high-speed video camera (MotionXtra NR4S2, DEL Imaging Systems, Cheshire, CT). High-speed video clips were recorded at 4,000 images/second with a resolution of 256 horizontal x 512 vertical pixels. Illumination was provided by a 300-W xenon light source (PS-NP1; Polarion, Seoul, Korea). Then, from the images of the vocal fold with the maximal glottal area, the glottal gap areas were measured using ImageJ software.

3. Results and discussion

3.1 Preparation and Characterization of PAAM/Gelatin (PG) Hydrogel

First, we synthesized thermo-responsive, self-healing and tissue adhesive IPN hydrogel by integrating two types of polymers: PAAM and gelatin (Figure 1). PAAM hydrogel can be synthesized from copolymerization of acrylamide and bisacrylamide in distilled water by free radical polymerization using Ammonium persulfate (APS), an oxidizing agent, and tetramethylethylenediamine (TEMED), a free radical stabilizer.[39, 40] In this study, gelatin solution was mixed at a temperature of 60°C with acrylamide (AAM) and N,N′-Methylenebisacrylamide (MBAA) solution before initiating copolymerization with APS and TEMED. This process ensures the homogenous mixing of gelatin within PAAM hydrogel. Then, cooling down the temperature of hydrogel enables gelatin network to form a physical entanglement with PAAM network. After copolymerization and cooling process, PAAM/gelatin hydrogel (PG hydrogel) was successfully synthesized. As shown in figure 1a, physical entanglement between PAAM and gelatin allows maintaining flexible characteristics of PAAM in addition to self-healing property of gelatin after denaturation the characteristics of gelatin which has a self-healing property due to denaturation.[41] Therefore, broken gelatin chains from stretching PG hydrogel can be repaired by heating up and cooling down the hydrogel. Additionally, PG hydrogel can be synthesized easily in any shape with one-pot method by simply mixing PAAM and gelatin solutions together in the mold (Figure 1b). Therefore, PG IPN

hydrogel as an artificial vocal fold can be synthesized with a customized shape that would fit each patient. There were numerous studies of IPN hydrogels with specialized characteristics such as stretchability and self-healing property. For example, Sun et al. prepared an IPN hydrogel using alginate/Ca2+ ionic bonds that were very tough and stretchable, however, it did not have tissue adhesive property.[42] Darnell et al. developed a tough and biocompatible hydrogel with IPN of alginate and PAAM, nevertheless it could not replace natural tissue due to lack of self-healing ability.[13] There were several studies of IPN hydrogels with self-healing properties to restore its morphology such as agar/PAAM IPN hydrogels.[43-45] However, self-healing process of agar/PAAM hydrogel could be activated at a high temperature range of 90-100°C which is too high compared to human temperature and would be difficult to use for living organism. [46, 47]

After synthesis of PG hydrogel, we investigated characteristics of the hydrogel such as porosity, swelling ratio, and cytotoxicity before mechanical testing to investigate the possibility as an artificial vocal fold. In this study, as table 1 shows, four groups of PG hydrogel with different ratio of PAAM to gelatin (PAAM only group, 19:1 group, 9:1 group and 7:1 group) was synthesized to examine characteristics of hydrogels and to select proper hydrogel for the artificial vocal fold. PAAM-only group is PAAM hydrogel without the addition of gelatin, and 19:1, 9:1 and 7:1 groups represent the ratio of PAAM and gelatin. First, physical characteristics of hydrogels were observed before investigating its potential as a

vocal fold. Analysis with SEM confirmed successful copolymerization of acrylamide and bisacrylamide and existence of gelatin among 19:1, 9:1 and 7:1 groups. Presence of gelatin can be seen through somewhat crushed pore morphology compared to the PAAM-only image (Figure 2a). SEM images of lyophilized PG hydrogel were analyzed further with ImageJ for average pore area. As shown in figure 2b, as the proportion of gelatin in PG hydrogel increased, the average pore area of lyophilized hydrogel decreased. This demonstrates that main factor of pore area in lyophilized PG hydrogel depends on the PAAM network, but the increase in gelatin amount results reduced pore area due to complex physical entanglement between gelatin helix and PAAM. Eventually, this character affects the factors such as swelling behavior and elastic modulus of the hydrogel.

Then, swelling behavior of hydrogel was measured for forty-eight hours to investigate the sensitivity of hydrogel against the moisture in case of implantation for the vocal fold. The difference of swelling behavior was insignificant initially, but at the fully swollen condition, swelling ratio of hydrogel decreased as the amount of gelatin increased in PG hydrogel (Figure 2c). This can be explained by the lower water absorbing property of gelatin compared to that of PAAM. [48] After the swelling test, to confirm non-intentional broken chains were made during swelling test, swollen samples were lyophilized and reswelled with distilled water to measure reswelling ratio. Reswelling ratios of all hydrogel groups were almost the same as their first swelling ratios and showed the same phenomenon of increased gelatin

content lowering swelling property (Figure 2d). This demonstrates that although swelling or lyophilization may change the volume, it does not affect physical entanglement and network of polymers in PG hydrogel. Thus, a network of PG hydrogel is strong against swelling or lyophilizing process which shows its functional stability as an artificial vocal fold.

In order to see biocompatibility of using PG hydrogel as a vocal fold, cytotoxicity of the hydrogel was studied. Especially, for vocal fold implantation, cytotoxicity is critical since the biomaterial has to stay in the body for long period. Many biomaterials such as Teflon has been used as a permanent material for vocal fold augmentation, however, undesirable foreign body reaction was one of the concerns.10 Cell cytotoxicity of hydrogel was measured by culturing adipose-derived stem cells (ADSCs) derived from human adipose tissue for 3 days in gel-conditioned media. LIVE/DEAD assay confirmed about 99% cell survival in all groups of PG hydrogels, which show that combination of PAAM and gelatin is toxic-safe biomaterial to be used for vocal fold engineering (Figure 2e and 2f).

3.2 Mechanical and Rheological Properties of PG Hydrogel

To measure its capability to function as vocal fold tissue, PG hydrogels' mechanical and rheological properties were studied. As shown in figure 3a, stress-strain curve graph from compression test demonstrated that yield point of PAAM only and 19:1 groups started at around 70% of strain while yield point of 9:1 and 7:1

groups started at around 60% of strain. Additionally, stress-strain curve graph from tensile test showed as strain increases, required stress for PAAM only and 19:1 groups become larger than that of 9:1 and 7:1 groups (Figure 3b). From compression and tensile test data, the result confirms that the decrease of gelatin in PG hydrogel causes hydrogel to be stiffer. This phenomenon can be explained by less PAAM network, which implies the lower amount of copolymerization of acrylamide and bisacrylamide, resulting in reduced stiffness. The elastic modulus of PAAM-only, 19:1, 9:1 and 7:1 groups from compression test were calculated to be around 8 kPa, 7.5 kPa, 5.4 kPa and 5.1 kPa (Figure 3c). The different elastic modulus for different PAAM and gelatin ratio suggest PG hydrogel can function as an applicable biomaterial for various types of tissues with simple volume ratio change in PAAM and gelatin. From analyzing mechanical test results, we found PAAM/gelatin ratio with mechanical properties suitable to serve as vocal fold tissue. Especially, 9:1 and 7:1 groups have same Young's modulus of human vocal fold tissue which is 3.9 – 5.7 kPa. [49, 50] Then, stretch-ability was measured in order to find the most stretchable PG hydrogel group which is an essential factor in producing phonation via a vibration in vocal fold. As shown in figure 3d, stretch-ability of PAAM-only and 19:1 groups were measured to be around 500% and showed the negligible difference between these groups. For 9:1 group, stretch-ability was increased up to around 700%. This result shows that reduced the complex network of PAAM which eventually allows greater stretch-ability. On the other hand, stretch-ability of 7:1

group was measured to be around 600%, which is higher than PAAM-only and 19:1 groups, but lower than 9:1 group. These results suggest an increase in gelatin proportion enhances stretch-ability. However, exceeding (excess) amount of gelatin in PG hydrogel weakens the PAAM network which eventually causes hydrogel to be ruptured easily during stretching. Overall, 9:1 PG hydrogels have mechanical properties highly resembling that of vocal fold tissue.

Additional amplitude sweep test, frequency sweep test, and thixotropic-loop tests were performed to investigate rheological properties and stabilities of PG hydrogels. Amplitude sweep test was carried out from 0.01 % to 100 % of shear strain at a constant angular frequency in order to see any deformation of PG hydrogel during a change of strain. As shown in figure 3e, all PG hydrogels have a higher storage modulus (G') than loss modulus (G'') which is a characteristic of viscoelastic solid. Also, as gelatin proportion in PG hydrogel increases, storage modulus decreases while loss modulus increases. This shows an increase of gelatin proportion leads to increased viscoelastic liquid property in PG hydrogels. From the range of 0.01% to about 20% of shear strain, all PG hydrogels were stable. However, after 20% of shear strain, as strain increased, storage modulus and loss modulus started to decrease. At 100% of shear strain, storage modulus was about 400-450 Pa. However, even at 100% of shear strain, G' of all PG hydrogels was higher than G'' which represents that there was no deformation of hydrogels. Although it was a minor difference, after 20% of shear strain, the storage modulus of 9:1 group was higher

than that of 19:1 and 7:1 groups. This shows 9:1 group maintains the most stable network against deformation among groups. Frequency sweep test showed a similar pattern as amplitude sweep test did. Although the difference between G' and G'' became smaller as frequency increased, deformation of PG hydrogel did not occur from 1 Hz to 100 Hz of oscillatory frequency (Figure 3f). After 25 Hz of frequency, the storage modulus of 9:1 group was higher than that of 19:1 and 7:1 groups confirming the stability of 9:1 group even in high oscillatory frequency. Lastly, thixotropic-loop test with alternating 3% and 80% of shear strain was carried out to analyze shape recovery behavior of PG hydrogel. Even after the 80% of shear strain, PG hydrogel recovers its shape and modulus in less than 6 seconds (Figure 3g). The same result is shown in repeated oscillatory strain. Recovering ability is one of the crucial factors for vocal fold engineering because an artificial vocal fold should endure deformation without losing its stability during high-frequency vibration for phonation. Therefore, with its remarkable recovery ability in severe hear strain conditions, PG hydrogel can serve as long-term vocal fold implant. Additionally, rheological properties of canine vocal fold were analyzed from amplitude sweep test to confirm the feasibility of using PG hydrogel as an artificial vocal fold (Figure 3h). Storage modulus values at a shear strain of 0.5, 1.0, 1.8 and 3.2% were measured to examine rheological similarity between PG hydrogels and vocal fold. As figure 3h shows, 9:1 group has the most similar rheological behavior as vocal fold has compared to 19:1 and 7:1 groups. Especially, at 3.2% of shear strain, the rheological

similarity between 9:1 PG hydrogel and vocal fold was about 95% which suggests 9:1 group to be the best candidate for the artificial vocal fold. Mechanical and rheological data demonstrate that PG hydrogel, especially 9:1 PG hydrogel, is suitable for vocal fold tissue engineering due to its similar mechanical and rheological properties as vocal fold tissue, in terms of excellent stability, fast recovery and resistance to deformation under high strain.

3.3 Self-Healing Ability of PG Hydrogel

PG hydrogel has self-healing ability due to temperature dependent denaturation property of gelatin. To test its self-healing ability, hydrogels were cut into pieces, re-attached under pressure and kept for an hour at the 60℃ chamber. When hydrogels were cooled down to room temperature, hydrogels were self-healed to its original form (Figure 4a). In addition, the same result of self-healing ability was observed with different sizes of the hydrogel as well (Figure 4b). This can be explained by the movement of gelatin in the 60℃. Based on the second law of thermodynamics, free statistical random coils produced from denatured gelatin triple helices at high temperature gains mobility.[51, 52] As hydrogel cools down, gelatin freezes and reversible triple helix structure re-network.[53] During this phase, new physical entanglement between PAAM and gelatin occurs inducing self-healing effect of PG hydrogel.

To examine the mechanism of self-healing in microscale, we synthesized two types of PG hydrogel: 1) PG hydrogel that contains gelatin attached to fluorescein isothiocyanate (FITC-gelatin) and 2) normal PG hydrogel without FITC. Then, the two different PG hydrogels were attached and conducted self-healing tests to observe whether FITC-gelatin can form a new network with normal PG hydrogel without FITC during self-healing process. As shown in figure 4c, FITC-gelatin was found in normal PG hydrogel which demonstrates that all PG hydrogel groups have successful self-healing ability. In addition, quantitative analysis of confocal laser scanning microscopy (CLSM) image confirmed that intensity of FITC-gelatin decreased, as penetration distance in the normal PG hydrogel increased. In other words, the amount of FITC-gelatin near contacting region of two hydrogels was larger than the amount of FITC-gelatin far from contacting region (Figure 4d). In addition, this demonstrates that FITC-gelatin from PG hydrogel was able to move to the normal hydrogel during the self-healing process. To confirm the result, we repeated same self-healing test of PG hydrogel with rhodamine B isothiocyanate (RITC) attached gelatin (RITC-gelatin) and the test showed the same result of self-healing as a case of using FITC-gelatin (Figure 4e). RITC-gelatin was able to move to the ordinary PG hydrogel during self-healing process and it was confirmed quantitatively as figure 4f shows. This self-healing effect of PG hydrogel will serve as a huge advantage when applied to vocal fold engineering. For example, when the implanted artificial vocal fold is torn or damaged by an accident, biomaterials

without self-healing effect cannot function as intended requiring another surgical removal and implantation which is a burden for both patient and healthcare professionals. Compared to other gels with self-healing abilities such as gels containing agar, temperature required for them to self-heal is around 90-100°C which is implausible in human body.46 On the other hand, self-healing temperature for PG hydrogel is only about 40℃. Therefore, it would be easy to handle, less time consuming for clinicians, safe and less of a financial burden for patients to repair vocal fold.

3.4 Tissue Adhesion of PG Hydrogel via the Anchoring Effect

After examining self-healing effect of PG hydrogel, we also found that PG hydrogels have remarkable tissue adhesiveness to be developed as an implantable vocal fold. Nowadays, different kinds of adhesives are used for comprehensive applications in the medical area such as artificial implantable biomedical device, tissue repair, and hemostatic patch.[54-58] However, adhesives have many drawbacks. For example, an adhesive such as cyanoacrylate is cytotoxic, and commercial adhesives such as fibrin glue TISSEEL (Baxter) and commercial polyethylene glycol-based adhesives, DURASEAL (Confluent Surgical), adhere weakly in wet environments. [54] For the artificial vocal fold to function well in the human body with moisture, the material must have strong adhesion with the vocal fold which vibrates as fast as 1,000 Hz in a moisturized environment. Therefore,

adhesiveness of PG hydrogels was tested. As shown in figure 5a, the hydrogel tightly adhered to the porcine skin. Tough adhesiveness of PG hydrogel was possible due to adhesive characteristics of PAAM materials [13, 54, 59-61] and the penetration of gelatin network into tissue from PG hydrogel. For instance, when the hydrogel adheres to tissue, gelatin network from PG hydrogel penetrates to the tissue and creates an anchor by forming another physical entanglement with polymers in the tissue such as collagen. Simply, this anchoring effect along with adhesiveness of PAAM reinforces tough adhesiveness by forming a network not only inside, but also outside of the hydrogel.

In order to observe the role of gelatin as an adhesive, PG hydrogel was immersed in distilled water at room temperature to release gelatin from the hydrogel. As shown in figure 5c, the largest gelatin release was observed in 7:1 group which released a total of 293 μg in 48 hours. Other 19:1 and 9:1 hydrogels showed similar behaviors and released approximately 81 μg and 186 μg of gelatin, respectively. Also, it was observed that about half of gelatin was released in the first hour of immersion. This released gelatin can penetrate into the tissue and strengthen the adhesion between hydrogel and tissue.

To verify the penetration of gelatin into tissue for adhesive application, PG hydrogel with FITC-gelatin adhered to the porcine skin and the interface of contacting area between hydrogel and tissue was studied by CLSM. Anchoring effect

was confirmed from FITC-gel found in tissue which also proves gelatin from PG hydrogel penetrated into the tissue (Figure 5b). The intensity of fluorescence faded as a region of the tissue became far apart from the contacting area of tissue and hydrogel (Figure 5d). Same tests and analysis with CLSM were repeated with all groups including 19:1, 9:1 and 7:1, and results confirmed gelatin from PG hydrogel penetrated into tissue successfully which resulted in tough adhesiveness via anchoring effect.

Then, we focused on possible improvements that can be made in adhesiveness of PG hydrogel for it to serve as an artificial vocal fold. In order to endure numerous vibration and perform phonation, PG hydrogel has to show strong adhesion to the tissue. Therefore, despite its tough adhesiveness from anchoring effect, we tried to enhance adhesive property even stronger so it can be utilized as an artificial vocal fold without any complicated procedure such as another chemical fixation or suturing during implantation. There were many options in making adhesion stronger. However, one condition that was indispensable was accessibility; the material should be simple and easy to use like commercially available materials. Silica particle was an excellent candidate that satisfied this condition. Thus, silica solution (Ludox TM-50 colloidal silica, Sigma-Aldrich) was applied on the surface of the hydrogel before attaching it to the tissue (Figure 5e). Attached silica particles increase contacting surface area between the hydrogel and tissue.[55, 62] In other words, increase of contacting surface area elevates frictional force which results in

higher adhesion force.[55] Therefore, anchoring effect from gelatin of PG hydrogel along with adhesiveness of PAAM creates a synergistic effect with increased contacting surface from silica particle to yield tougher adhesiveness. To evaluate the improved adhesion energy of PG hydrogel with silica particle, adhesion tests proceeded with 180-degree peeling test (Figure 5f). As shown in figure 5f, PG hydrogel with silica particle exhibited stronger adhesion than PG hydrogel only group. More specifically, PG hydrogel with silica particle group required more than twice the force applied to peel PG hydrogel-only group (Figure 5g). After the peeling test, SEM analysis of detached PG hydrogel confirmed that silica particles were well distributed and well connected with gelatin and PAAM (Figure 5h). Thus, application of silica particle in PG hydrogel would enhance adhesion for implantation and function as durable vocal fold without any accidental detachment.

An additional experiment was carried out to investigate the strong adhesiveness of PG hydrogel with silica particles on the wet tissue by testing its adhesion to decrease blood loss from in porcine heart. Normal PG hydrogel (hydrogel) and PG hydrogel with silica particle (hydrogel with SP) were applied as patches to seal the large defect in the porcine heart (Figure 6a). As shown in movie S1, both of hydrogel groups were able to stop bleeding for some time and even when bleeding reoccurred, it was less severe than that of negative group. Furthermore, the duration of which the hydrogel with SP stopped bleeding reached almost 3 minutes. Then, the average blood loss was measured at every 30 seconds for quantitative data.

The result showed that both hydrogel and hydrogel with SP groups are good candidates for the hemostatic application. In hydrogel group, no bleeding occurred for the initial 30 seconds, and when bleeding reoccurred, average blood loss of hydrogel group was up to one-fifth of that of negative group (Figure 6b). 30 seconds of complete hemostatic effect is a huge benefit especially for cardiac surgery because even 10 seconds of bleeding in heart ruins the clear view of the surgical area with blood. Additionally, it will give enough time for the surgeon to prepare for a more permanent hemostatic treatment.[63] Total blood loss of hydrogel group was about 3.4 ml whereas that of the control group was about 9.8 ml (Figure 6c). Although 30 seconds of hemostatic effect is enough, hydrogel with SP group showed less blood loss (0.3 ml of blood loss in 3 minutes), and no blood loss until 150 seconds. These demonstrated that the blood loss was significantly reduced by the adhesion of both hydrogel groups compared to the negative control. Especially, tissue adhesion of PG hydrogel with SP was confirmed to be strong to be used as a hemostatic patch. Therefore, making it a suitable candidate for vocal fold implantation without going through any additional process, such as suturing to adhere tissue and the hydrogel.

3.5 Application of PG Hydrogel : Artificial Vocal Fold

Based on the mechanical, rheological and biocompatible test results, we tested the 9:1 PG hydrogel as an artificial vocal fold, an organ that produces phonation and determines the pitch of the sound. Thus, we set up ex vivo canine

vocal fold model and implanted the hydrogel as an artificial vocal fold tissue to investigate its functionality and potential. We had a total of three experimental groups: normal vocal fold as native, resected, and hydrogel implanted vocal fold as an artificial vocal fold (Figure 7a). In order to investigate its phonation abilities, measurement and analysis of vocal fold vibration were carried out using a high-speed camera (Figure 7b).[64, 65] Vibration and opened glottal areas of the vocal fold was measured as shown in figure 7c during air flow from the vent to the vocal fold. For the resected vocal fold group, there was no vibrational movement during the air flow. However, when we applied the hydrogel onto the tissue, it vibrated with a vertical phase difference between upper and lower part of the vocal fold (movie S2). Glottal area waveforms and displacement values of hydrogel implanted group showed similar trends to those generated by the native group, particularly for within-larynx comparison. Hydrogel implanted group vibrated with a maximum-minimum glottal area of around 17,000 pixels which is more improvement compared to that of the resected sample that showed the maximum-minimum glottal area of around 4,500 pixels, as well as reduced glottal area magnitude and waves excursion (Figure 7d). This result suggested that there was some recovery of vocal tissue that matched the mechanical and rheological properties of the native group. Although PG hydrogel maximum-minimum glottal area values slightly match that of the normal vocal fold tissue, its functionality from high-speed camera showed the great potential as a medical device which can be a solution for the patients who cannot produce

phonation due to scar change of vocal fold or vocal fold resection due to disease like laryngeal cancer.

Then, we studied dehydration resistance of PG hydrogel to see how long it could last in the larynx when it was implanted as a medical device. Hydrogels were dehydrated in the 37°C chamber which was the same temperature as the human body. As shown in the Figure S1, at low humidity, all groups of hydrogels were dehydrated rapidly by day 15 and showed the indistinguishable difference of dehydration resistance. However, at intermediate humidity, all groups of hydrogels maintained about 80 % of mass at day 40 and showed high dehydration resistance (Figure 7e). This suggests that if the hydrogel were to be implanted as a medical device, PG hydrogel attached with human tissue in larynx would have consistent moisture from the tissue, and this would result in slower dehydration than the samples from the dehydration test. Thus, the test results confirm that PG hydrogels with mechanical, rheological and strong dehydration resistance can be applied as an artificial vocal fold in larynx for long period without the need for replacement.

4. Conclusion

In this study, we have described the development of a PAAM/gelatin based IPN hydrogel (PG hydrogel) that can be used as an artificial vocal fold. The characteristics of PG hydrogel demonstrate safe biocompatibility, highly adjustable mechanical and rheological properties. Additionally, this biomimetic hydrogel shows great self-healing ability, dehydration resistance and tough tissue adhesion that can overcome challenges for vocal fold engineering. Based on these properties, the functionality of PG hydrogel as a vocal fold was tested and the result shows successful functional restoration of the ex vivo vocal fold defect model. Although it would require further modification and research such as clinical tests, PG hydrogel with its characteristics mentioned shows great potential to be utilized in vocal fold engineering.

The composition of PG hydrogel				
Sample name	PAAM only	19:1	9:1	7:1
Total solid (g/ml)	0.15	0.1475	0.145	0.14375
PAAM (g/ml)	0.15	0.1425	0.135	0.13125
Gelatin (g/ml)	0	0.005	0.01	0.0125

Table 1. Composition information of PG hydrogels tested

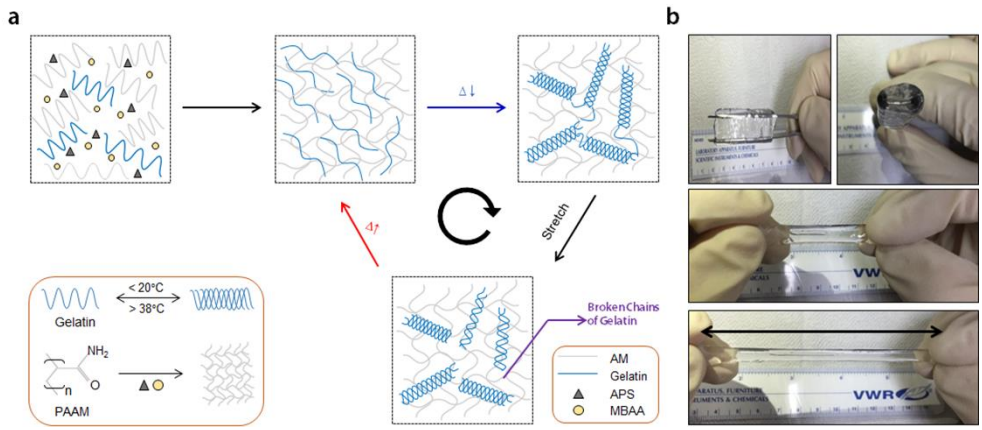

Figure 1. Design of PAAM / Gelatin (PG) hydrogel.

(A) SEM Thermo-responsive and self-healing PG hydrogel was synthesized by mixing two different polymers (polyacrylamide and gelatin). PAAM hydrogel was synthesized from copolymerization of acrylamide (AM) and N,N'-Methylenebisacrylamide (MBAA) by free radical polymerization using ammonium persulfate (APS) and tetramethylethylenediamine (TEMED). PG hydrogel was synthesized forms a physical entanglement between gelatin and PAAM. **(B)** Photographs of PG hydrogel shows the various shape of transparent hydrogels. Cylindrical shaped PG hydrogel was stretched to show its stretchability and flexibility.

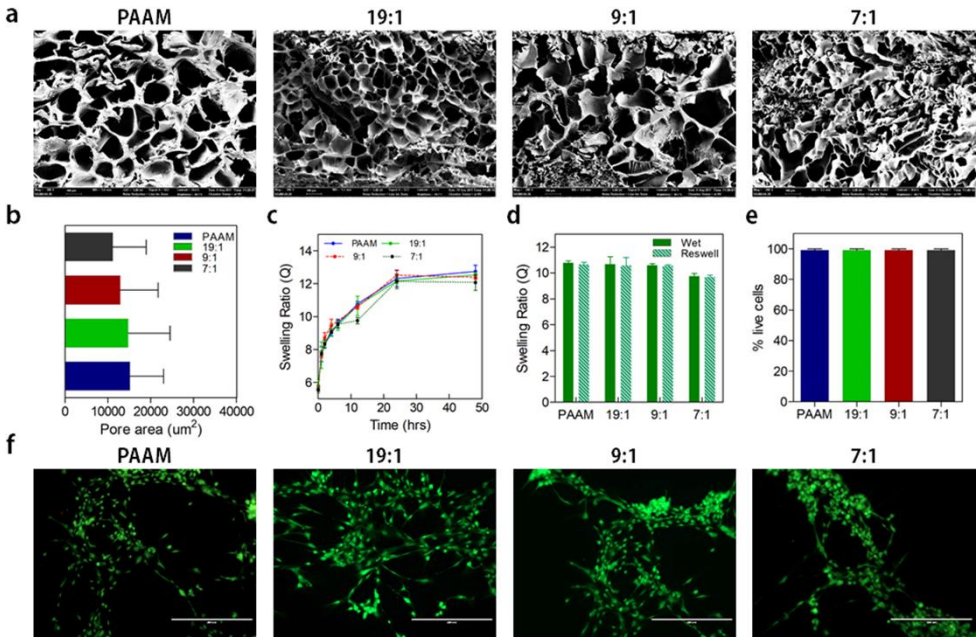

Figure 2. Characterization of PG hydrogel with various combination of PAAM and gelatin concentration.

(A) FESEM images of PG hydrogel groups **(B)** Average pore area of lyophilized PG hydrogel with different proportion of PAAM and gelatin. **(C)** Swelling behavior of hydrogel groups (n=3) **(D)** Comparison between swelling ratio and reswelling ratio of four PG hydrogel groups. (n = 3). **(E)** Live/Dead staining of mouse mesenchymal stem cells cultured for 3 days in gel (PAAM, 19:1, 9:1, 7:1)-conditioned media. Live cells stain green and dead cells stain red. EVOS; mag 10x; scale bar = 100 μm. **(F)** Viability of mouse mesenchymal stem cells cultured for 3 days in gel (PAAM, 19:1, 9:1,7:1)-conditioned media (n = 3). Error bars indicate SD.

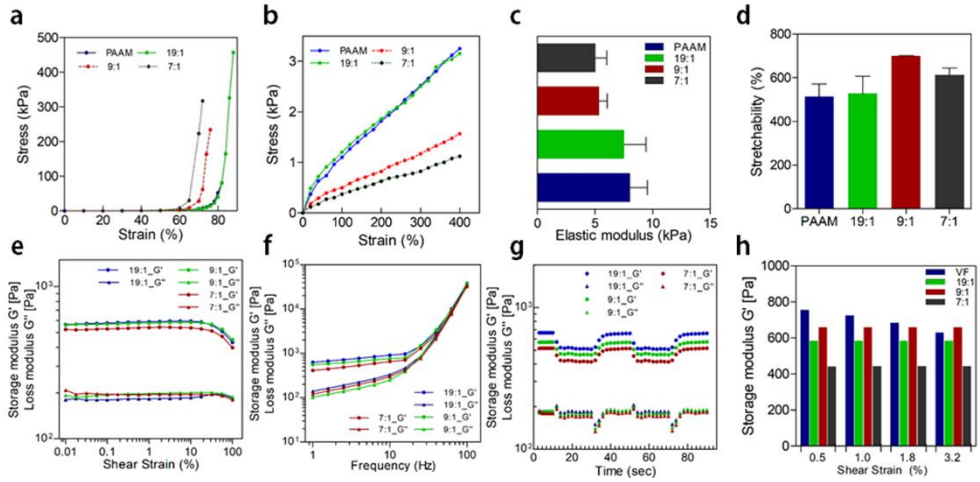

Figure 3. Mechanical and rheological properties of PAAM / gelatin (PG) hydrogel.

(A,B) Comparison of mechanical properties of PG hydrogel groups from **(A)** compression test and **(B)** tensile test. **(C)** The elastic modulus of PG hydrogel groups from a compression test. **(D)** Stretch-ability of PG hydrogel groups **(E,F,G)** Storage modulus (G') and loss modulus (G'') of PG hydrogels on **(E)** amplitude sweep test, **(F)** frequency sweep test and **(G)** thixotropic-loop test. **(H)** Rheological similarities between the vocal fold and PG hydrogels from amplitude sweep test. Error bars indicate SD;

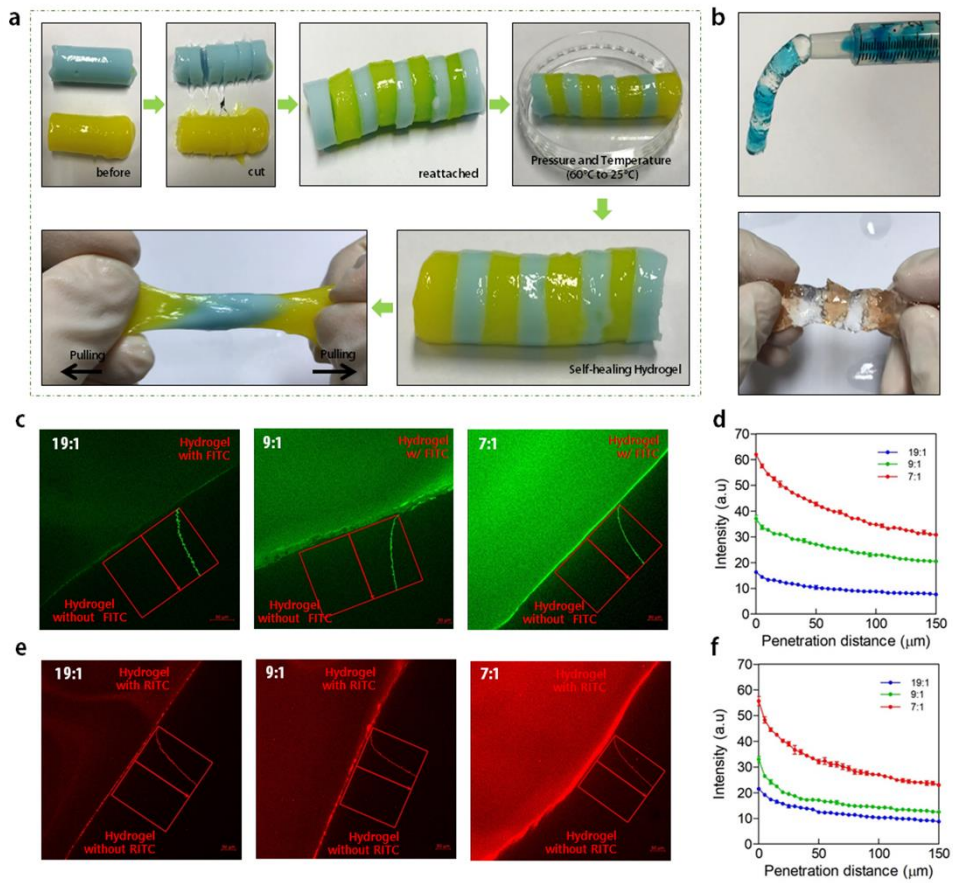

Figure 4. Self-healing properties of PG hydrogel

(A) Self-healing test of PG hydrogel. **(B)** Self-healing property from different sizes of PG hydrogels **(C)** CLSM images of self-healing interface between a PG hydrogel with FITC-gelatin and a PG hydrogel without FITC-gelatin. mag 20x; scale bar = 50 μm. **(D)** Intensity of penetrated FITC-gelatin into a PG hydrogel without FITC-gelatin. **(E)** CLSM images of self-healing interface between two hydrogels (RITC). Mag 20x; scale bar = 50 μm. **(F)** Intensity of penetrated RITC-gelatin into a PG hydrogel. Error bars indicate SD; N = 4

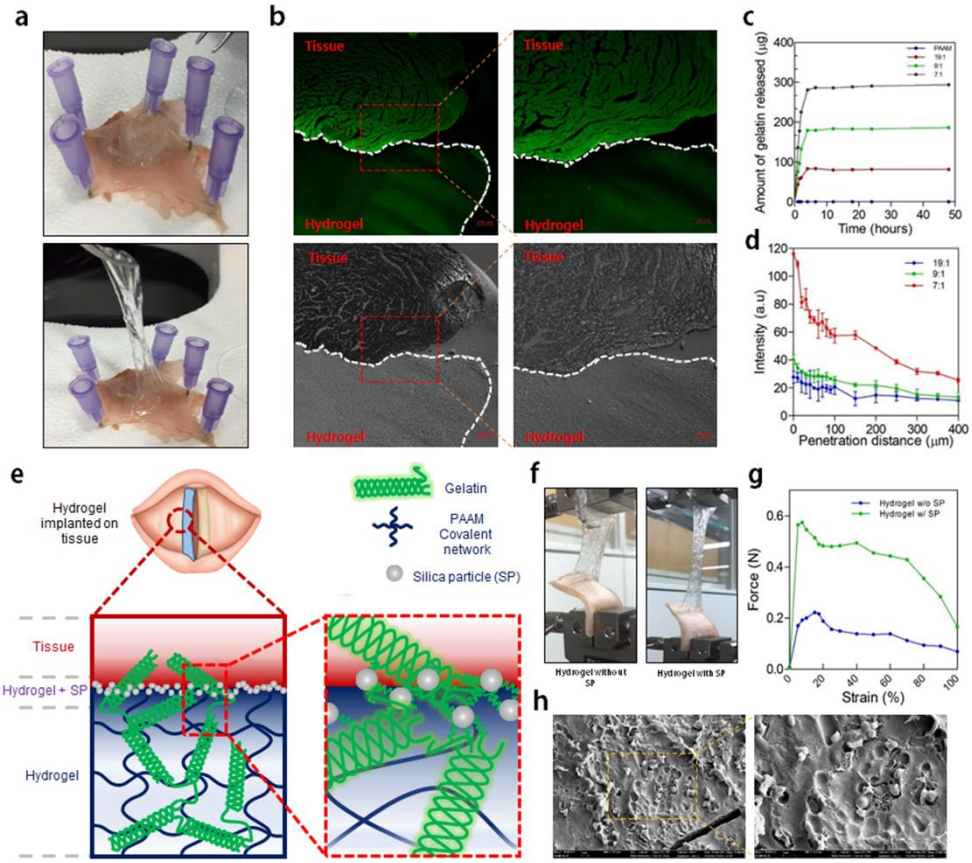

Figure 5. Adhesion of PG hydrogel

(A) Adhesion effect of PG hydrogel to the porcine skin. **(B)** CLSM images show the interface of contacting area between PG hydrogel with FITC-gelatin and cardiac tissue of a porcine. (Left image: mag 20x; scale bar = 50 μm, Right image: mag 10x; scale bar = 100 μm). **(C)** Amounts of gelatin released from different PG hydrogel groups were measured for a time duration of 50 hours. **(D)** Intensity of penetrated FITC-gelatin from PG hydrogel groups to cardiac tissue of a porcine. **(E,F,G,H)**

Enhanced adhesion of PG hydrogel with silica particles (SP). **(E)** The scheme shows the interface between the tissue and PG hydrogel with SP. **(F)** Photographs show the peeling test of hydrogel group and hydrogel with SP group against the porcine skin. **(G)** The graph shows how much force required to peel per change of strain from hydrogel group and hydrogel with SP group. **(H)** FESEM image of hydrogel with SP after the peeling test against the porcine tissue. Error bars indicate SD; N = 3

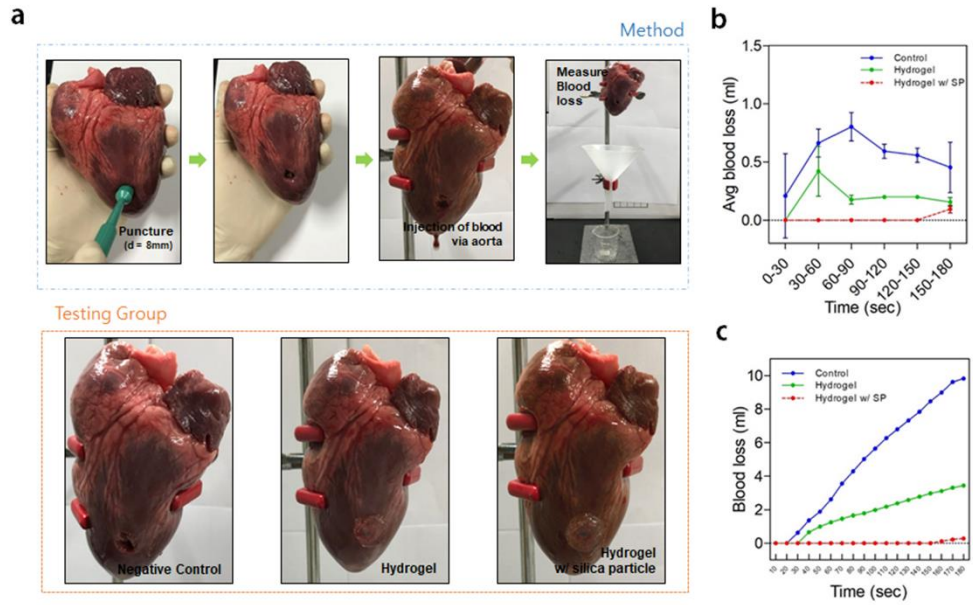

Figure 6. Hemostatic test by adhesion of PG hydrogel

(A) Testing group and procedure of hemostatic test by adhesion of PG hydrogel. Testing groups were negative control, hydrogel, and hydrogel with a surface covered by silica particles. **(B,C) (B)** Average blood loss for every 30 seconds and **(C)** cumulative blood loss with time duration of 180 seconds were measured to compare the adhesion effect of three testing groups. Error bars indicate SD; N = 3

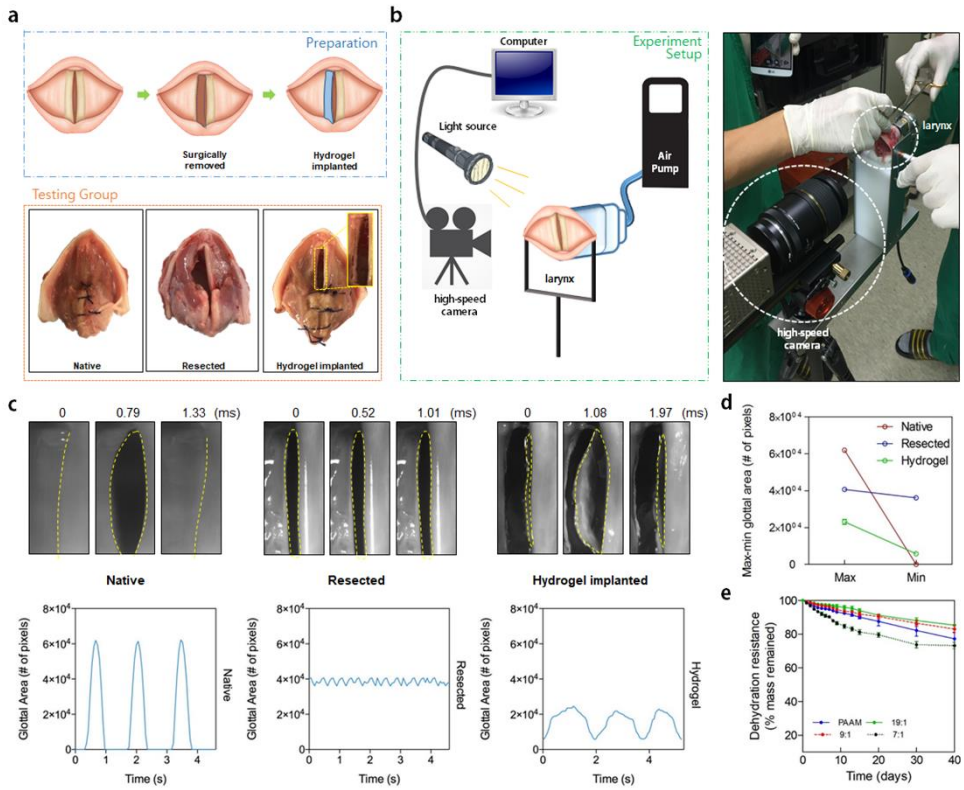

Figure 7. Application of PG hydrogel: vocal fold.

(A) Sample preparation and a testing group of stroboscopy for PG hydrogel vocal fold. **(B)** Scheme of the experimental setup of stroboscopy for larynx that was implanted with PG hydrogel **(C)** Photographs of PG hydrogel vocal folds from stroboscopy shows the opening region of vocal fold during air flow from the vent. The corresponding graphs represent the opening glottal area and its patterned frequency of vocal fold. **(D)** Maximum and minimum glottal area of testing groups during stroboscopy. **(E)** Dehydration resistance of PG hydrogel groups (n = 4).

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요약 (국문초록)

음성 회복을 위한 자기 치유 및 접착성 인공 조직 하이드로겔

후두암으로 인한 성대 절제술 후 목소리의 상실은 삶의 질이 낮아지는 등의 심각한 문제들을 야기한다. 이를 극복하기 위해 여러 방법들을 통한 음성의 기능적 복원이 시도되었지만, 진동을 통해 음성을 생성하는 성대의 유연성과 같은 독특한 기계적 특성으로 인해 음성 회복에는 여전히 많은 문제들이 남아있다. 이에 이 논문에서는 폴리아크릴아마이드와 젤라틴의 비율을 최적화하여 성대 조직의 고유 물리적·기능적 특성을 지닌 인공 조직 하이드로겔을 개발하였다. 본 연구의 하이드로겔은 성대와 유사한 기계적 특성과 더불어 높은 생체적합성과 탈수 조건에 강한 저항성을 보였다. 또한 이 폴리아크릴아마이드/젤라틴 하이드로겔의 자가 치유능은 성대에 이식시 지속력을 보강해주었고, 견고한 접착성은 성대 조직에 안정한 부착을 가능하게 했다. 마지막으로 본 연구의 하이드로겔을 개과 동물 모델의

성대에 직접 이식함으로써 음성기능의 회복을 확인하여, 개발된 하이드로겔이 성대 유사 인공조직으로 활용될 수 있다는 것을 입증하였다.

주요어: 폴리아크릴아마이드, 젤라틴, 하이드로겔, 인공 성대, 자가치유, 접착성, 조직 공학