공학석사 학위논문

Ultra-fast self gelling and wet adhesive hemostatic powder based on chitosan catechol and oxidized hyaluronic acid

카테콜-키토산과 산화 히알루론산 기반의 파우더형 지혈제

2023년 2월

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이 논문을 공학석사 학위논문으로 제출함

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Abstract

Hemorrhage is the most common preventable cause of death on a battlefield, and the second leading cause of death in civilians worldwide. Even if the patient survives, restricted oxygen supply to the tissues can lead to serious sequelae by organ failure. Therefore, an effective hemostatic agent with a fast reaction is required for emergent bleeding. Herein, we fabricated a fast gelling and adhesive powder for the hemostatic agent based on Catechol-chitosan(CHI-cat)/oxidized hyaluronic acid(oHA). By absorbing liquid(water, blood, exudates, etc.), the aldehyde group of oHA and the amine group of CHI-cat reacted by the Schiff-base reaction and formed a robust cohesive network in situ. Also, the optimal ratio of the catechol group showed strong adhesion to wet tissues. In the murine and porcine hemorrhage model(in vivo), the adhesive powder rapidly absorbed blood and stopped bleeding. This novel type of hemostatic powder could reduce the risk of the emergent bleeding situations and reduce death.

Keyword :
Hemostasis, Rapid gelling, Wet adhesion, Biocompatible, Antimicrobial
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Chapter 1. The Scientific Background

1.1. Hemorrhage and mechanism of Hemostasis

The only fluid connective tissue in the body, the blood, is composed of 55% plasma and 45% blood cells. Plasma consist mostly of water and contains electrolytes, hormones and plasma proteins such as fibrinogen, globulin, albumin, and prothrombin. Blood cells are composed of red blood cells, white blood cells, and platelets. Blood is regarded as a fluid connective tissue due to these live cells suspended in the plasma. Blood receives oxygen from the lungs and transports it to cells throughout the body, and release carbon dioxide generated as a result of cellular respiration outside the body. Aside from carrying nutrients and hormones to tissues, blood also carries waste products to the kidney and liver, where they are processed and removed. And, blood helps maintain homeostasis by regulating the internal body temperature, respiration, body fluid and pH. It promotes the wound healing process by blood clotting in the site of wound, and also plays a role in protecting body against pathogens through phagocytosis of white blood cells.

Bleeding is a state in which blood that should be inside has leaked out due to damage of blood vessels for various causes such as injury and disease. Uncontrollable bleeding is still a leading cause of death in emergency, hospitals, and battlefields.[1−3] Massive bleeding can lead to death, and even if

survives, various complications can occurred such as neurological damage, hemorrhagic shock and multiple organ failure caused by decreased blood volume and oxygen supply to the tissue for a long time.[4]

Bleeding initiates a hemostasis that naturally occurring physiological process to halts bleeding from injury site. The term "Hemostasis" which refers to the phenomenon of blood stagnation, is derived from the ancient Greek words "haíma" which means "blood", and "stasis", which means "stoppage".[5] When blood vessel damage occurs, blood vessels constrict in order to minimize the bleeding. Additionally, platelets gather at the site of the bleeding to create a platelet plug, a process known as "primary hemostasis.".[6] Simultaneously coagulation factors are activated and fibrin mesh was deposited over the bleeding site to form strong clot that can stop bleeding, which process known as "secondary hemostasis". [7, 8] This "secondary hemostasis" process can be divided into an intrinsic and extrinsic pathway. A small amount of bleeding can be coagulated by the hemostatic mechanism in the body, but severe or uncontrolled blood loss due to damage of large blood vessels or multiple trauma, the natural coagulation pathway alone is not enough to hemostasis.

Chapter 2. Ultrafast self-gelling and wet adhesive hemostatic powder based on Chitosan catechol and oxidized hyaluronic acid

2.1. Introduction

In emergency situations, such as disasters, mass hemorrhage is one of the leading causes of death. According to U.S army statistics, the overall mortality rate decreased due to the development of field first aid throughout the Vietnam War, the Gulf war, and the Iraq war, but the proportion of bleeding deaths increased.[9, 10] Bleeding accounts for 91% of preventable causes of death, and if first aid is delayed, oxygen supply to tissues will be delayed for a long time, causing serious aftereffects. Bleeding is one of the leading cause of death not only in combat and disaster but also in medical sites. With the development of medical technology, the performance of surgical operations increased, and the frequency of bleeding during surgery also increased.[11] In particular, in the case of a patient with diseases related to blood coagulation factors, there is a limit to treating them with various existing hemostatic agents.[5]

The most ideal treatment for bleeding is to supplement the amount lost with the same amount of blood until the time when red blood cells and platelets are reproduced in the body. For blood transfusions, various pre-tests including blood compatibility tests must be carried out and a sufficient amount of blood must be stably stored. Therefore, in a battlefield, a sudden disaster, or an emergency, blood transfusion is practically limited. So, hemostasis to prevent further bleeding is most important in the on-site first aid stage before being transferred to a medical facility where medical treatment such as blood transfusion is possible. Physical methods include directly compressing the bleeding area or using hemostatic gauze that combines chemical components such as chitosan or kaolin. In addition, a method of using mechanical tools such as a tourniquet or a clip is still widely used in emergency. There is also a cold and hot method of controlling the rate of outflow of blood by lowering the temperature of the bleeding site or electrically burning the blood vessels to prevent further bleeding. Chemical sanctions such as convergent agents, blood coagulants, and local hemostatic agents are also being used, and recently methods using biomolecules such as chitosan, hyaluronic acid, and polyacrylate are being actively researched.

However, there are limitations in using the above various types of hemostasis methods in emergency sites. Gauze, used for direct compression, promotes hemostasis by aggregating blood coagulation factors or platelets by chitosan or kaolin components,

which form a blood clot within minutes to suppress bleeding. Anyone can easily use it by applying gauze to the bleeding site and compressing it with a compression bandage, but use of areas where compression is not possible is limited, and for patients with problems with blood coagulation mechanism such as platelet disorders, it is difficult to expect effects other than direct compression. Chemical hemostatic agent based on blood coagulation sanctions are difficult to store and use stably under harsh conditions such as emergency or combat sites due to their short storage and low temperature storage conditions. In addition, most of them are hydrogel-type and have poor physical properties and adhesive strength, so it is difficult to apply to pulsating bleeding such as aortic damage, and if not maintained properly, problems such as secondary bleeding may occur.

Currently, the military is using tourniquets, hemostatic gauze, and pressure bandages as components of combat first aid kit(IFAK : improved first aid kit). In most cases of mass bleeding, where pressure is limited or hemostasis is restricted by gauze, tourniquet is recommended, and to prevent further tissue damage, it must be removed within 4 hours of use. X-stat in US army is the syringe type hemostatic agent that the sponge inside sucks in blood and swells to stop bleeding by compressing the wound. It can be useful for intracavitary bleeding such as penetrating injury, but it has the disadvantage that the degree of expansion cannot be controlled, so excessive pressure can cause damage to the surrounding tissue.

And also, like the tourniquet, there is a hassle that must be removed after 4 hours of use using radiography or the like. There are various hemostats such as clamps used for bleeding from large blood vessels such as the pelvic cavity, but there is a limit to the combatants possessing all of the materials, and there is a difficulty in mastering the use of various types of hemostats.

An ideal hemostatic agent should be able to satisfy both convenience, safety, and hemostatic ability. It is easy to carry and use, so it can be used in any field, and anyone should be able to use it easily with simple education. It should be safe to use inside and outside the body as it has no side effects and is biocompatible and hemocompatible. It should be stable, long-term, and can be stored regardless of temperature conditions, so that it can be used even under harsh conditions. In addition, sufficient physical properties and adhesion capabilities should be secured so that rapid hemostasis is possible and it can be stably applied to mass bleeding.[12, 13] To develop an ideal hemostatic agent, we paid attention to hyaluronic acid and chitosan, which are bio-based polymers, and catechol derived from mussels proteins which have wet adhesiveness.

Chitosan is known to promote blood coagulation by attracting erythrocytes with electrostatic attraction, and has an muco-adhesiveness. Hyaluronic acid, an ECM component, has high water retention capacity, and is widely used for wound healing because of its anti-inflammatory effect.

However, these two polymers lack adhesiveness in wet condition to be used as hemostatic agents. In a wet condition, water interferes with adhesion causing plasticization, swelling, erosion, or hydrolysis of the polymer, preventing the polymer from adhering to the site. In addition, the adhesion is weakened through interfacial wicking and interfacial crazing. To strengthen the adhesion is wet condition, we interested in aquatic organisms that are stably attached and survived in actual water. Mussels can survive by immobilizing themselves with specific adhesive pads, and the proteins attached to the adhesive interface are mainly composed of Mefp-3 and Mefp-5. The two proteins have an amino acid composition with a high ratio of tyrosine-modified dopa. In particular, the catechol functional group present in the chemical structure of dopa is responsible for this adhesive force. Catechol is a molecule in which two hydroxyl functional groups are bonded to a benzene hexagonal ring. It interacts with the surface in various bonding methods to show adhesiveness. It easily hydrogen bonds with oxide films, or improves adhesion with the surface through electrostatic interaction, metal coordination bond, and cation π interaction through metal-base cationic materials. In addition, when catechol is oxidized and transformed into quinone, it forms and irreversible covalent bond with the adhesive material, making the adhesive stronger. We improved the adhesive strength by conjugating a catechol group into chitosan.

In addition, we thought about the formulation part to improve adhesion and retention to the bleeding site. To adhere, the interaction between the functional group of tissue and the functional group of hydrogel must occur. And if there is interfacial water in between, it takes time for functional group of hydrogel to diffuse into interfacial water. In bleeding site, it is not simply wet conditions, but due the nature of blood vessels, it has a pulsating and flow out continuously. So, if it cannot be attached to the bleeding site quickly, hydrogel will be swept away by the blood flow. However, when applied in the form of powder, it absorbs interfacial water and interacts with the functional groups of the tissues, enabling adhesion within seconds.[14] Therefore, we tried to apply the above polymers in powder form.(Fig. 2.1.)

2.2. Materials and methods

2.2.1. Materials

Chitosan(75% deacetylation, M_w ≈ 50~190kDa), Sodium periodate($NaIO_4$), Ethylene glycol, Hyaluronidase, tert-butyl carbazate, Trichloroacetic acid, Gluta aldehyde were purchased from Sigma Aldrich(USA). Hyaluronic acid(M_w >1,000kDa) was purchased from Lifecore Biomedical(USA). 3,4-Dihydroxyhydrocinnamic Acid(Hydrocaffeic acid), 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)(DMTMM) were purchased from TCI(Japan). MES buffer, Lysozyme, TNBSA solution(2,4,6-trinitrobenzene sulfonic acid, 5% w/v) were purchased from Thermofisher(USA). MES buffer was prepared by dissolving 500mg of MES powder in 500ml of deionized and adjusted the pH to 5.0 with 1M HCl aqueous solution. Deuterium Oxide(D_2O) was purchase from Zeotype.

Dialysis tubing(M_w cut off 3~5kDa) was purchased from Thermofisher(USA). Calcein AM and Ethidium homodimer were purchased from Invitrogen(USA). Citrated sheep blood were purchased from MB cell(Korea).

2.2.2. Synthesis and characterization of Oxidized Hyaluronic acid(oHA)

Oxidized Hyaluronic acid(oHA) was synthesized according to the previous studies with slight modifications.[15, 16] Hyaluronic acid(HA, 1g) was dissolved in distilled water (100ml, 1w/t%) and sodium periodate($NaIO_4$) in distilled water was added dropwise into the HA solution under stirring. The molar ratio of sodium periodate to HA was 1:1, 1:0.5, and 1:0.25. The mixture was reacted for 2 ~4 hours under the dark conditions at RT(room temperature). Ethylene glycol was added to the mixture to stop the oxidation reaction. Then, the mixture solution was dialyzed against distilled water for 3 days to remove unreacted $NaIO_4$, the water needs to be changed every 8 hours. After 3 days, the dialyzed solution was lyophilized and we obtained the final product which was like a white sponge type.

The molecular weight of oHA was analyzed using High Performance Liquid Chromatography(HPLC)(Ultimate 3000, Dionex, USA). The functional group was analyzed through an infrared spectrum obtained through a resolution of $4cm^{-1}$ and 32 scans in the $4000-750^{-1}$ frequency range using Bruker's FTIR(TENSOR27, Bruker, Germany). The aldehyde substitution rate(oxidation degree) was calculated by TNBSA(2,4,6-Trinitrobenzenesulfonic Acid) assay. A 5mM oHA and a 10mM of

tert-butyl carbazate in 1% aqueous tri-chloroacetic acid were mixed and reacted for 24 hours, and then 1ml of 0.02% TNBS was added and reacted for 1 hour. After 1 hour, 50ul of reacted solution and 50ul of 0.5N HCL were put into 96-well plate, and the absorbance at 335nm was measured using a microplate reader(Infinite m200 pro, TECAN, Switzerland).

2.2.3. Synthesis and characterization of Chitosan catechol(CHI-cat)

Hydrocaffeic acid was conjugated to Chitosan via DMTMM coupled reaction following the method reported elsewhere.[17] First, chitosan(1g) was dissolved in MES buffer(pH 5.0, 1 w/t %, 100ml). Hydrocaffeic acid(0.5g) was dissolved in MES buffer and DMTMM(0.74g) in MES buffer was added dropwise into the Hydrocaffeic solution. The Hydrocaffeic acid/DMTMM mixture was added dropwise to the chitosan solution at RT(room temperature), under nitrogen conditions. The mixture was reacted for 12h under nitrogen conditions at RT. After 12 h, an excessive amount of ethanol was poured into the mixture to get the precipitate by filtration. Wash the precipitation by ethanol 2~3 times to eliminate unreacted hydrocaffiecacid and dissolved it in distilled water adjusting pH 5.5 using 1M HCl. The solution was freeze-dried. Then, we obtained chitosan catechol(CHI-cat) as the final product.

Lyophilized CHI-cat was dissolved in a D_2O solvent(< 1 w/t%) and then analyzed the degree of chemical composition and catechol group substitution by the ^1H-NMR. The functional group was analyzed through an infrared spectrum obtained through a resolution of $4cm^{-1}$ and 32 scans in the $4000-750^{-1}$ frequency range using Bruker´s FTIR(TENSOR27, Bruker, Germany).

2.2.4. Fabrication of Hemostatic powder

Freeze-dried oHA and CHI-cat were pulverized into powder using a freeze-mill machine(SPEX 6875D, SPEX, USA) and mortar. Each sample was in the same form as a sponge during freeze-drying, but when pulverized, it became a fine powder. Each powder was evenly mixed at a certain weight ratio. (CHI-cat/oHA : 0.5, 1.0, and 2.0) The particle size and shape of the powder were observed through microscope(EVOS M700, Thermofisher, USA) and SEM(JSM-7800F Prime, JEOL Ltd, Japan) images. While observing the particles under a microscope, PBS was treated to confirm the process of gelation. To confirm the structure of crosslinked network, treating with excessive PBS on each weight ratio of powder, sufficiently hydrated for 1 hour to make a hydrogel. After lyophilizing hydrogel, SEM images were taken to confirm the difference in structure between before and after hydration.

2.2.5. Gelation Kinetics analysis

Rapid gelation of powder via Schiff base reaction was verified using various methods. First, 20mg of powder was placed in a 10ml vial, treated with PBS, and flipped upside down to confirm the sol-gel transition of the powders over time.

The changes in functional groups of hydrogel caused by the formation of imine bonds via Schiff base reaction were analyzed using the FTIR spectrum(TENSOR27, Bruker, Germany).

To prove the rapid gelation quantitatively, TNBSA assay was used by slight modification. 5mM oHA and 5mM CHI-cat powder were mixed evenly, distilled water was treated to cause hydration, and then TNBSA 0.02% was treated after each time point. (0 ~ 180 sec) After 1 hour of treating TNBSA 0.02%, the reaction solution was centrifuged at 12,000g for 10 minutes, then the supernatant was removed. The reacted hydrogel was sufficiently dissolved by 0.5 N HCl, and 100ul of the solution was measured for absorbance at 335nm using a microplate reader(Infinite m200 pro, TECAN, Switzerland). The gelation time was verified by determining the number of remaining amine groups that did not react with aldehyde groups.

2.2.6. Rheological analysis for mechanical property

The dynamic viscoelasticity of the hydrogel was measured using a rheometer(ARES-G2, TA instruments, USA). To understand the

sample's deformation behavior within the non-destructive deformation range, an amplitude sweep test was performed. After loading the sample on the plate, hydration was performed using PBS, and storage and loss modulus were measured while adjusting from the 0.01 to 100% range. The frequency value was fixed to 10 rad/s. A frequency sweep test was conducted to explain the time-dependent behavior of the sample within the non-destructive deformation range. After loading and hydrating the sample on the plate in the same manner as above, the storage and loss modulus were measured while adjusting the frequency in the range of 10^{-1} ~ 10^{2} rad/s. The strain value was fixed to 1%. It was performed by adjusting the weight ratio of CHI-cat and oHA.

2.2.7. Adhesive property of powder

Adhesiveness to skin tissue was conducted using Lap shear test (ASTM F2255). Hemostatic powder was applied on 2.5 * 1cm size with hydrated porcine skin cut into 2.5 * 7.5cm, and then the other side was fixed with porcine skin. In that state, the shear stress was measured by pulling at a speed of 5mm/min using mechanical testing machine(UTM, Shimadzu, Japan). Shear stress was divided by the area of the powder sample applied to calculate adhesion strength. Adhesion strength was repeatedly performed while adjusting the weight ratio. The average value was derived by

repeatedly measuring at least three times for each experimental group.

A burst pressure test was performed according to the previous paper. A hole was made on the collagen tube using a 19G needle, and then blocked it with the powder. Using a three way valve, the collagen tube was connected to a faucet and manometer. The pressure was measured while filling the collagen tube with water. The maximum pressure at the time when the pressure rapidly decrease was measured as the burst pressure at which the hemostatic powder could withstand. The group that did not treat hemostatic powder was used as a control group, and the average value was derived by repeated measurements more than three times.

2.2.8. Swelling ratio of powder

100ul of PBS was dropped onto the 20mg of powder to fabricate hydrogel. Pre-fabricated hydrogels were placed in a 12-well plate, treated with PBS(2ml), and reacted at RT for 24h. After 24h, the excess PBS was wiped out with filter paper. Then, the wet hydrogel was weighed and compared to the dry condition to determine swelling ratio. The weight of the dry condition was measured after lyophilizing the wet hydrogel.: Where M is the wet hydrogel mass after 24 hours of hydration and M_0 is the mass after lyophilization of hydrogel.

$$\% \text{ swelling ratio } = \frac{[M_0 - M]}{[M_0]} \quad [18]$$

2.2.9. In vitro degradation test

CHI-cat/oHA powder was placed in a 12-well plate and then PBS(2ml) containing 0.1U hyaluronidase and lysozyme(1mg/ml) was added to the powder.[19-22] The plate was shaken(50rpm) at 37℃ for 24 hours. After incubating 24 hours, the solution was completely removed, and then the hydrated powder was rinsed with distilled water and lyophilized. By comparing the weight loss of the powder with the initial dry weights, the degradation ratio was calculated : where $W_{d(0)}$ is the initial dry powder mass and $W_{d(t)}$ is the dry powder mass after 24 hours.

$$\% \text{ Degradation(t)} = \frac{[W_{d(0)} - W_{d(t)}]}{W_{d(0)}}$$

2.2.10. In vitro biocompatibility test

NIH/3T3 cells were seeded in a 96-well plate(10,000 cells/well) and then incubated with the conditioned medium overnight. CHI-cat/oHA powder was dissolved in PBS(1 w/t%). The above solution was diluted to 0.1, 0.01 and 0.001% by growth medium. Diluted CHI-cat/oHA solution was added to each well followed by 24 and 48 hours incubation at 37℃ containing 5% CO_2. Cell viability was assessed using the Live/Dead cell staining method. After co-incubation for 24 and 48h, the added solution was removed and the

cells were washed with PBS. Culture media containing live/dead cell staining solution (Calcein-AM/Propidium Iodide) were added to each well and incubated for 15 min. After incubation, culture media were removed and cells were washed 2-3 times with PBS. Live cells were stained green by Calcein-AM and dead cells were stained red by Propidium Iodide. Growth medium without powder was used as a positive control.

2.2.11. In vitro antibacterial test

For confirming antibacterial ability of CHI-cat/oHA powder, we use E.coli and S.aureus as gram negative and positive bacteria. Adding 1ml of E.coli and S.aureus suspension(1×10^5 CFU ml^{-1}) each into 50mg of CHI-cat/oHA powder. And the samples were shaking-incubated at 37℃ for 12h, 200rpm. The bacteria-only solution was used as a positive control. After overnight incubation, the absorbance value of the supernatants was measured at 600nm. The calculation for the relative bacterial viability is shown below.: where OD_s is the absorbance of the samples and OD_c is the absorbance of the control group.

$$\text{Bacterial viability}(\%) = \frac{[OD_s]}{[OD_c]} \times 100$$

Then 100ul of the above suspensions were diluted with LB, spreading onto agar plates, and incubated for 12h at 37℃. The antibacterial ability was verified by comparing the degree of E.coli and S.aureus colony formation for each sample.

2.2.12. Hemocompatibility test

The hemolysis activity of powder was tested according to reported works. Whole blood was centrifuged (200 xg, 10 min) to separate the erythrocytes, which were then washed three times with PBS and diluted to 5% (v/v) in PBS. 10mg of powder was pre-hydrated by 100ul of PBS under incubation for 1h, at 37℃. After hydration, we eliminated the PBS and treated erythrocytes stocks(1,000ul), then incubated at 37℃ for 1h. Additionally, 100ul 1x PBS and 0.1% Triton X-100 were used as a negative and positive control, respectively. Following 1 hour, the erythrocyte suspensions underwent a 15-minutes centrifugation(500xg), and the supernatants were placed into a 96-well transparent plate. The absorbance of the solution at 540nm was measured by microplate reader. The hemolysis ratio was determined as follows :

$$\text{Hemolysis (\%)} = \frac{[OD_{sample} - OD_{negative}]}{[OD_{positive} - OD_{negative}]} \times 100$$

2.2.13. In vitro blood coagulation test

Adding 0.1M calcium chloride aqueous solution into citrated blood, and vortexing for 10s. Then, 10mg of a hemostatic powder, was added into the eppendorf tube containing 500ul above blood. Each eppendorf tube was turned upside down at predetermined time intervals. Blood clotting time was defined as the point at which uniform, stable clots began to form in the eppendorf tube upon

inversion. The blood clotting time was defined as the point at which there was no blood flow upon inversion.[23]

2.2.14. Blood cell adhesion on powder

To understand the hemostatic mechanism of the CHI-cat/oHA powder, we confirmed how well erythrocytes and platelets adhere to the powder. Treating whole blood and PRP(platelet-rich plasma) on the powder and incubating at 37℃ for 2 h, it was washed with PBS and fixed with gluta-aldehyde for 2 hours. After fixation, dehydration was performed with 50, 60, 70, 80, 90, and 100% ethanol for 10 minutes each. The dehydrated sample was dried at 37℃, coated with platinum, and photographed by SEM(JSM-7800F Prime, JEOL Ltd, Japan).

2.2.15. In vivo hemostasis test

The use of animals in this study received IRB approval, and all experimental procedures were carried out in accordance with the relevant ethical standards. The hemostasis of powder was assessed using a mouse bleeding liver model(C57BL/6, 80g, female).

A mouse's liver was exposed after an abdominal incision, and pre-weighted filter paper was placed underneath the liver. A part of the liver was punctured by a 19G needle to induce bleeding, and 50mg of powder was deposited on the site to stop bleeding. The

control group underwent no therapy after bleeding. To quantify the amount of lost blood, we weighted the filter paper before and after absorbing blood.

Rat liver and tail vein hemorrhage were employed as models to further show the powder's quick hemostasis. After puncturing the liver and cutting the tail vein, 100mg of powder was immediately applied on the bleeding site. Lost blood was quantified in the same way as above.

The bleeding spleen of porcine was used to demonstrate rapid hemostasis of powder also. 200mg of powder was administered right away to control the bleeding after incision. By weighing the gauze that had been wrapped around the incision site, the quantity of blood loss was measured. As a control group, a chitosan-based hemostatic gauze currently used in the military was used.

2.2.16. Statistical analysis

For the statistical studies, GraphPad Prism8 software was used. All data are presented as mean and standard deviation(SD). Statistical significance was performed by the Students t test for the 2 sets of data and one-way ANOVA for the 3 or more groups of data. Values of $P<0.05$ was regarded as statistically significant. The significance level is indicated as follows : * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$.

3. Result and Discussion

2.3.1. Preparation and characterization of CHI-cat and oHA

Chitosan is a polysaccharide obtained from the deacetylation of chitin, and is soluble only in an acidic pH environment.[24, 25] But the solubility of chitosan increase in a neutral-basic pH environment when the acetyl group is substituted with a catechol group. [26] Chitosan catechol (CHI-cat) was created by conjugating hydrocaffeic acid to the main amino groups in chitosan using 4-(4,6,-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM) for activating carboxylic acid group with carbodiimide.(Fig. 2.2 A) [17, 27]

The typical peaks for the functional groups of phenolic structures appeared at 1289, 820, and 780 cm-1, according to the FTIR spectra. The distinctive amine group absorption peak at 1590 declined, but the amide type 2 absorption peak at 1530 rose.(Fig. 2.3 A) This indicates that the amino groups on chitosan interacted with the catechol to generate amide. [17] [25, 28, 29] ^{1}H-NMR spectra represented the catechol protons (3H) in the 6.5-7.0ppm range.(Fig. 2.3. B) [27, 28, 30] The degree of substitution with catechol groups was calculated by dividing the integration value of the catechol proton peaks(6.5~7.0ppm) by the value of the acetyl group protons(1.9~2.0ppm, 3H) considering deacetylation rate. Although there were some differences between batches, an

average substitution degree of around 10% was obtained when chitosan, DMTMM, and hydrocaffeic acid were reacted at the same molar ratio(chitosan:DMTMM:hydrocaffeic acid=1:1:1). As the molar ratio increased, the substitution degree of catechol also increased(Chitosan:DMTMM:hydrocaffeic acid= 1:2:2, substitution degree : 20%).

Oxidized hyaluronic acid is formed by the ring-opening reaction of sodium periodate.(Fig 2.2 B) The molecular weight of hyaluronic acid inevitably decreases as the ring open reaction proceeds. At first, the solubility is low and the viscosity is very strong, but after the ring-open reaction, the viscosity is significantly lowered.[31] Oxidized hyaluronic acid confirmed the formation of an aldehyde group through FTIR spectra, and the peak in the 1605cm^{-1} region became stronger.(Fig 2.4. A) The degree of oxidation according to the reaction molar ratio was confirmed through TNBSA assay, and it was confirmed that slight difference occurred compared to the actual theoretical expected value.[32, 33] In order to confirm the degree of molecular weight reduction, GPC was performed, and it was confirmed that the decrease in molecular weight increased as the reaction time and the amount of sodium periodate increased.(Fig 2.4. B)

Considering the mechanical property of powder, the molar ratio of the HA and NaIO4 was 1:0.25 and the 2 hours reaction time were optimized as synthesis condition.

2.3.2. Preparation and Characterization of hemostatic Powder

The lyophilized forms of oxidized hyaluronic acid and chitosan-catechol were ground into a powder form using liquid nitrogen. The two powders were blended at a constant weight ratio to finally make a hemostatic agent powder. Also, the powder can be compressed and used as a stick type. (Fig 2.5 A,B) In order to confirm the particle size of each hemostatic powder and the changes during gelation, SEM, and FTIR spectra were confirmed.

The condition before and after hydration was compared through SEM images. In the powder state before hydration, uniform particle size was exhibited in all weight ratios, and in the hydrogel state after hydration, a dense network structure was exhibited. (Fig. 2. 5. C)

Rapid gelation of the powder is achieved by Schiff base reaction. (Fig 2.6. A) When powder particles are exposed to PBS, a Schiff base reaction occurs between the aldehyde group of oxidized hyaluronic acid and the catechol group of catechol-chitosan while quickly absorbing PBS, which can also be confirmed through the inverting test and microscope images. (Fig 2.6. B, C)

The formation of imine bonds by Schiff base reaction can be verified through FTIR spectra. It was confirmed that the imine bond was formed through the increase in the peak of the 1643cm^{-1}. (Fig 2.6. D) In order to confirm how quickly the Schiff base reaction

occurs, a modified TNBSA assay was performed. Using the principle that TNBSA reacts with an amine group to change color, it was confirmed how quickly the amine group of the powder decreases, and inversely, the degree of the amine group participating in the reaction was verified. As a result of the assay, it was confirmed that more than 30% of the amine groups took part in the reaction within 10s, and more than 50% of the amine groups took part in the reaction within the 30s.(Fig 2.6. E) Through this, it was found that the Schiff base reaction occurred within a few seconds, promoting the gelation of the powder and enhancing the mechanical properties.

2.3.3. Mechanical property of the Powder

Appropriate mechanical properties must be secured in order for the hemostatic agent to be applied to the bleeding site and stably attached and maintained. To verify this, amplitude sweep and frequency sweep were measured using a rheometer.

As a result of performing an amplitude sweep test to confirm the deformation behavior in the non-destructive deformation range, it can be seen that the gel maintains its structure firmly within the LVE region. The storage modulus of all powders in the LVE region was greater than the loss modulus, indicating that they were viscoelastic solid structures. In addition, the storage modulus showed a tendency to increase more and more as the ratio of CHI-cat increased. (Fig 2.7. A)

A frequency sweep test was conducted to measure the long-term stability of the gel, and it was found that the gel had a high yield strength of 10^2 or higher within the entire frequency range, indicating that it was a solid structure. Also, the stability of the structure was verified through the fact that G' value hardly changed within the entire frequency range. (Fig 2.7. B)

As a result of measuring the storage modulus for each weight ratio, it showed a tendency to increase as the proportion of CHI-cat increased. The storage modulus of CHI-cat/oHA(2.0) was 7kPa, which was higher than when CHI-cat was used alone(3.7kPa). (Fig 2.7. C)

The swelling ratio is affected by the structure of hydrogel, and the denser the network, the smaller the space that can contain moisture, reducing the swelling ratio. As a result of comparing the swelling ratio of the three groups(0.5, 1.0, and 2.0 of CHI-cat/oHA), it was confirmed that there was no significant difference in all three groups. (Fig 2.7. D) Through this, it was confirmed that the particles of the powder became uniform during freeze-milling, and the network was formed very densely to form sufficient mechanical properties, and accordingly, the swelling ratio was not high.

Therefore, considering reological data and swelling ratio, the CHI-cat/oHA(2.0) group was set as the optimization condition for the hemostatic powder.

2.3.4. Degradability of the Powder

A degradability test was conducted to confirm the retention of the powder in the body. 0.1U of hyaluronidase was treated for 24 hours in consideration of the fact that the capacity of hyaluronidase usually used to decompose the hyaluronic acid filler is 20 to 100U, and most of it is decomposed within several hours. In addition, considering that chitosan is degraded by lysozyme, an enzyme in the body, lysozyme was treated at a concentration of 1mg/ml for 24 hours.

After 24 hours, the enzyme was removed, and after lyophilization, it was compared with the initial weight, and it was confirmed that there was a significant difference depending on the ratio of CHI-cat and oHA. When hyaluronidase was treated, it was degraded from a maximum of 60% to a minimum of 30%.(Fig 2.8. A) In the case of lysozyme, it was confirmed that the decomposition was from a maximum of 60% to a minimum of 20%.(Fig 2.8. B) As the ratio of CHI-cat increased, the degree of degradation decreased.

Considering the level of enzyme concentration and half-life in the body, it was confirmed that the CHI-cat/oHA(2.0) group was stably maintained in the body and, if necessary, could be decomposed through high-concentration hyaluronidase treatment.

2.3.5. Adhesion property of the Powder

Adhesion is achieved by various functional groups of CHI-cat and oHA. The aldehyde group of oHA and the catechol and amine group of CHI-cat are bonded to various functional groups of tissues through covalent and non-covalent bonds.(Fig 2.9)

To confirm the adhesiveness of hemostatic powder, porcine skin hydrated with PBS was attached to the slide-glass while the powder was applied to see how quickly it adhered. It was confirmed that the porcine skin is adhered to by the hemostatic powder and lifted against its own weight within 2 seconds. (Fig 2.10. A) In addition, in order to verify the adhesion persistency of the powder, after 10 and 30 minutes, the holding power was checked by twisting the skin or flowing water. It was confirmed that stably adhered to the skin without falling off even after twisting or washing with water. (Fig 2.10. B)

The hemostatic powder showed stable adhesion not only to the skin but also to various surfaces such as brass and ceramics. It showed strong adhesive strength that could stably lift a weight of about 300g. (Fig 2.11.A) In addition, as a result of applying the powder while the porcine lung was incised, it was confirmed that the lung function was stably maintained without additional air leakage. It was confirmed that the powder did not fall off and was stably adhered to even if the incision site was pulled.(Fig 2.11.B)

A lap shear test was performed to quantitatively confirm the adhesive strength of the hemostatic powder. The powder was applied to the porcine skin which was hydrated with PBS while pressing the other side to the porcine skin and attaching it, and the adhesive strength was measured while pulling the skin using UTM.(Fig. 2.12.A) It was confirmed that the adhesive strength increase as the CHI-cat ratio increase based on oHA. It is known that the adhesive strength of commercially used such as fibrin glue is around 20kPa through previous studies. Considering that the adhesive strength in the optimized conditions(CHI-cat/oHA: 2.0) is 30kPa or more, it can be seen that it has very good adhesive strength. (Fig. 2.12.B)

At the bleeding site, blood is ejected in a pulsatile manner by the pressure applied to the vessel wall, the hemostat must be able to withstand this pressure. To verify this, a bursting pressure test was conducted.(Fig 2.12.C) Under optimized conditions, it was able to withstand 250mmHg. Considering that the average arterial pressure is 120~150mmHg, it was verified that it can be used sufficiently even for pulsatile bleeding. (Fig 2.12.D)

2.3.6. Biocompatibility and Hemocompatibilty of powder

Biocompatibility is a very important factor in hemostatic agents. In order to be applied to internal bleeding as well as external,

toxicity should not occur in vivo. To this end, in vitro cytotoxicity experiments were conducted. As a result of the cytotoxicity test, it was confirmed that there was no toxicity in all sections of 0.1 to 0.001%. It was confirmed that the material was biocompatible through cell growth without toxicity on Day 1 as well as Day 2.(Fig 2.13.A) As a result of quantitatively confirming cell viability based on Live/Dead images, all groups showed a high survival rate of more than 95%. (Fig.2.13.B)

Hemostatic agents are applied not only outside the body but also inside the body and come into direct contact with blood vessel and blood. Therefore, hemolysis should not occurred when applying. For blood compatibility, hemolysis rate must be under 5% for biomedical materials. To verify this, a hemolysis test was performed using erythrocyte diluted solution. Diluted erythrocyte solution was treated with each powder, incubated for 1 hour, and the absorbance of the supernatant was compared. Triton X was used as a negative control and normal saline was used as a positive control. Compared to the control, CHI-cat was slight higher than 5%, but CHI-cat/oHA treated blood samples was less than 5%. Although a slight color change occurred due to the oxidation of CHI-cat, it was found that hemolysis was rarely observed at less than 5%. (Fig 2.14)

2.3.7. Antibacterial property of powder

Chitosan is known to have an anti-inflammatory effect, and hyaluronic acid is known to have a bacteriostatic effect, although not a bactericidal effect. To demonstrate the antibacterial effect when these two were mixed, an experiment was conducted using E.coli and S.aureus. In the case of chitosan, it was confirmed that the antibacterial action does not occur in a neutral to a weakly acidic environment, but occurs only in an acidic environment. However, chitosan-catechol showed high antibacterial effects even in neutral conditions.(Fig 2.15 B, D) CHI-cat/oHA hemostatic powder showed excellent antibacterial effect through the absence of colony formation.(Fig. 2.15 A, C) Wounds are very vulnerable to infection in a humid environment. If the above hemostatic agent is applied, it can be prevented from such infection. Additionally, the powder can be kept stable and for a long time even under challenging situations, such those on the battlefield.

2.3.8. Hemostasis of the powder

In order to confirm the blood coagulation effect of hemostatic powder, in vitro blood clotting test was performed using sheep blood. As a result of measuring the blood clotting time of sodium chloride-treated citrated blood, it took 13~15min. Compared to the average blood clotting time of 7~9 min, it took slightly longer,

which is thought to be due to variables such as blood storage conditions and coagulation factors. It was confirmed that the blood clotting time was significantly reduced in all groups except for oHA treated group. It was confirmed that coagulation was not performed properly because oHA was dissolved while contacting blood. On the other hand, it was confirmed that CHI-cat rapidly absorbed blood while in contact with blood and quickly coagulated within 30 seconds while changing into a hydrogel form.(Fig 2.16 A, B)

It is known through previous studies that the functional group of CHI-cat promotes blood coagulation while forming a membrane with plasma proteins.[34] (Fig 2.20 A-B) It is also known that the positive charge of chitosan attracts negatively charged blood cells by electrostatic attraction and promotes rapid coagulation. This was also confirmed by SEM images. As a result of observing powder after treating whole blood and PRP(platelet-rich plasma), aggregated red blood cells and activated platelets were confirmed.(Fig. 2. 20 C-D) Through this, it was confirmed that this hemostatic powder is possible regardless of the blood coagulation factor, and this can be stably applied to patients with problems with coagulation factors.

The hemostasis property was confirmed in vivo. After causing bleeding by making a puncture in the liver using a 19G needle, the hemostatic powder was treated.(Fig.2.17 A) It was confirmed that the powder quickly adhered to the bleeding site, absorbed blood, and promoted coagulation to prevent further bleeding.(Fig

2.17.B) When the group not treated was used as a control, the control group had an average of close to 100mg of bleeding, whereas the group treated with powder showed a low blood loss of less than 10mg(mostly no bleeding), demonstrating the hemostatic ability.(Fig 2.17.C)

After inducing hemorrhage by puncturing the liver of the rat with the biopsy, the group treating hemostatic powder showed a higher hemostatic effect compared to the control group. (Fig 2.18. A~C). The model in which bleeding was induced by cutting the tail vein showed the same rapid hemostasis effect, and it was found that most of the hemostasis was achieved within 1 minute. (Fig. 2.18.D~F)

After incising the porcine spleen with a blade, bleeding was induced and hemostatic powder was applied to the bleeding site. As a control group, Hemoblock, a chitosan-based hemostatic gauze currently used in the ROK.army, was applied. Compared to the bleeding amount of 2000mg in the Hemoblock applied group, the powder allied group showed a high hemostatic effect with a bleeding amount of about 570mg. (Fig. 2.19.A-C)

Chapter 3. Conclusion

We fabricated a hemostatic powder based on chitosan-catechol and oxidized hyaluronic acid. Gelation occurs rapidly by the Schiff base reaction between the amine group of catechol chitosan and the aldehyde group of oxidized hyaluronic acid., and also it showed high adhesive ability through the interaction between various functional groups and tissue functional groups. In the form of a powder, it absorbs interfacial water between hemostatic agent and tissue, and acts quickly, showing high and stable mechanical properties and adhesive strength.

It showed an excellent hemostatic effect, which was also verified through in vivo experiments using mice, rats, and porcine. Since chitosan-catechol shows a hemostatic property by forming a membrane with plasma proteins and chitosan attracts blood cells by electrostatic attraction, it is applicable to those with blood coagulation issues. It has an antibacterial effect, so it can be stored and used stably even in harsh conditions.

It can be used in various forms by compressing the powder or fixing it to gauze through the backing, it is expected to be used in disaster and emergency sites of civil and military.

Figures

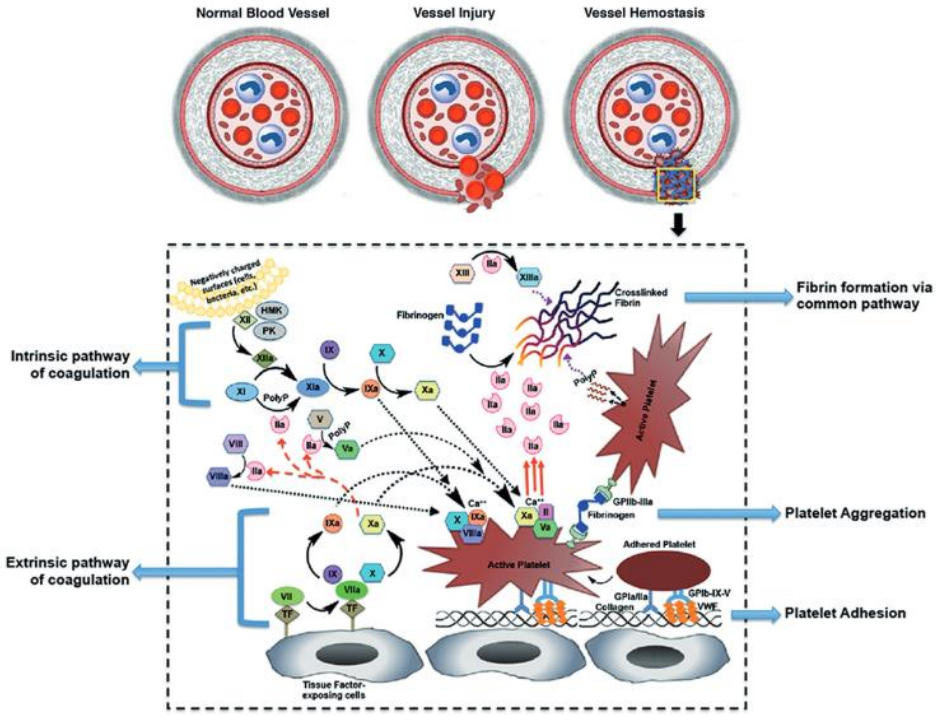


Figure 1.1 Scheme illustration of hemostasis mechanism(cascade pathway of coagulation) (copyright 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim and Adv. Mater. 2018)

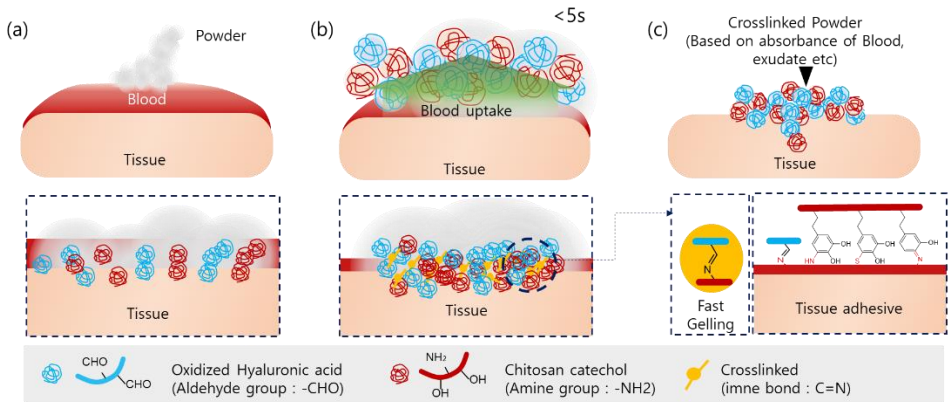
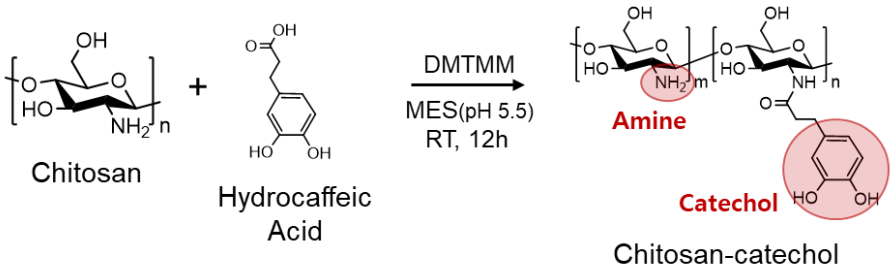


Figure 2.1 Overall scheme illustration of hemostatic powder based on catechol chitosan(CHI-cat) and oxidized hyaluronic acid(oHA)

The hemostatic powder was fabricated by mixing catechol-conjugated chitosan(CHI-cat) and oxidized hyaluronic acid(oHA). When the powder hydrated by water, it became hydrogel quickly by Schiff base reaction between the aldehyde group of oHA and the amine group of CHI-cat. And also, powder adheres to tissues very well by various functional groups of powder

A



B

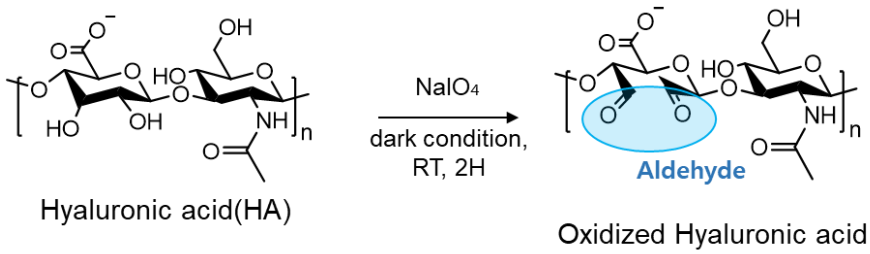
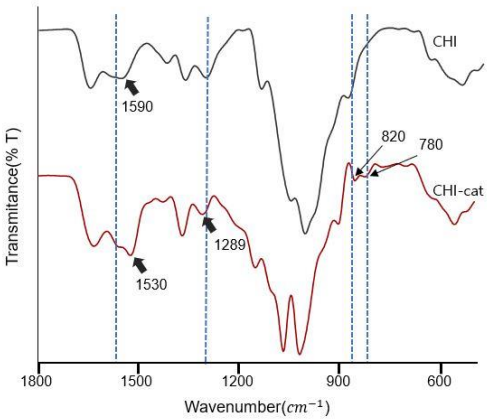


Figure 2.2 Scheme illustration of synthesis CHI-cat and oHA

(A) Chitosan and Hydrocaffeic acid were reacted to generate catechol conjugated chitosan(CHI-cat) via DMTMM reaction

(B) Hyaluronic acid and sodium periodate to generated oxidized hyaluronic acid(oHA) via ring-opening chemistry.

A



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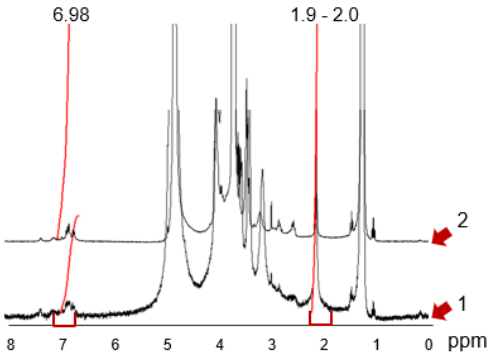
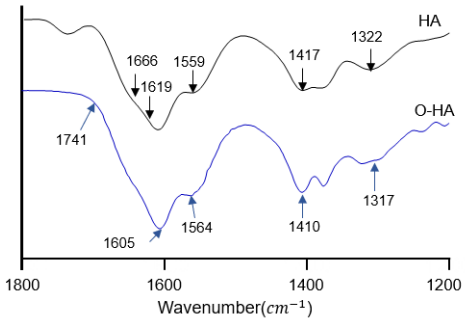


Figure 2.3 Characterization of CHI-cat

(A) FTIR spectra of CHI-cat.

(B) ^{1}H-NMR data of CHI-cat

A



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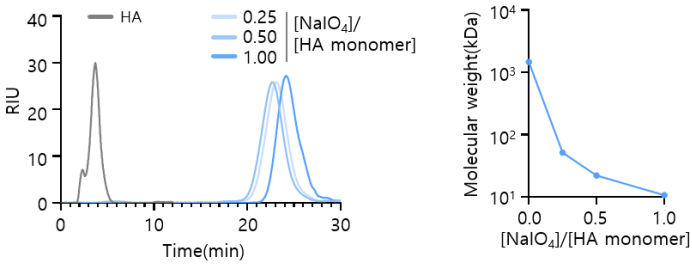
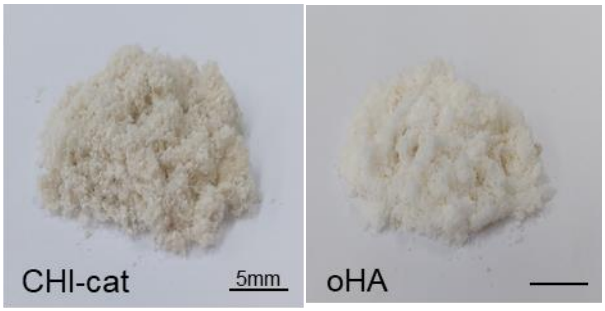


Figure 2.4 Characterization of oHA

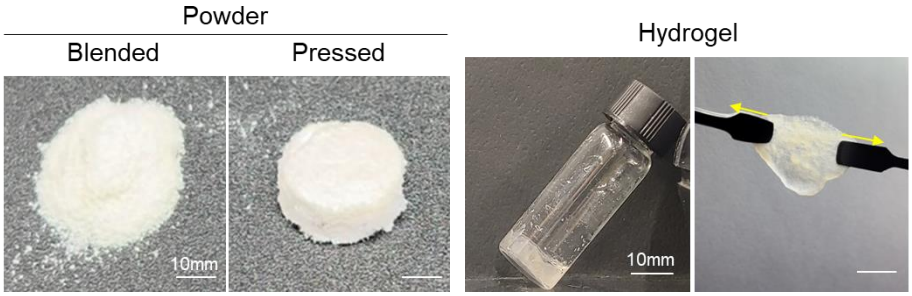
(A) FTIR spectra of oHA.

(B) GPC data of oHA

A


CHI-cat
5mm
oHA

B


Powder
Blended
Pressed
10mm
Hydrogel
10mm

C

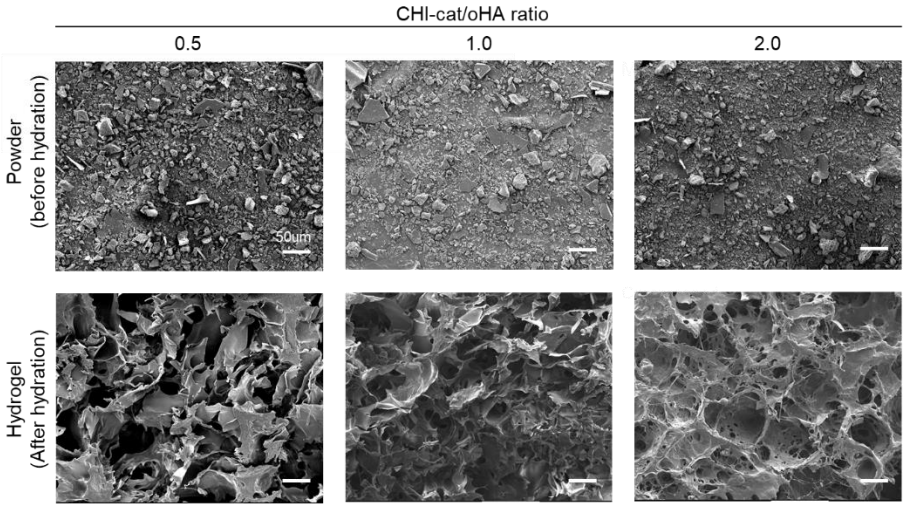
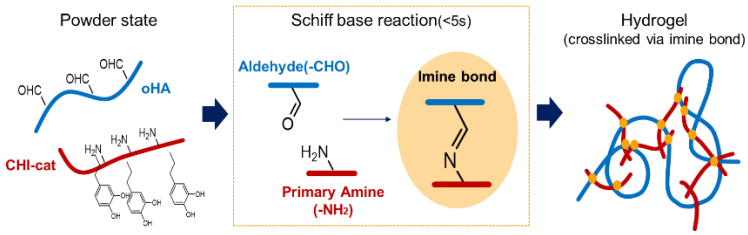

CHI-cat/oHA ratio
0.5
1.0
2.0
Powder
(before hydration)
50um
Hydrogel
(After hydration)

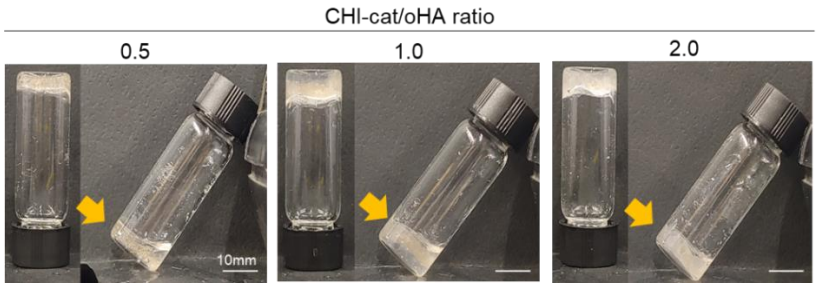
Figure 2.5 Fabrication of the hemostatic powder

(A) Photographs for CHI-cat and oHA powder after freeze milling (B) Photographs for hemostatic powder based on CHI-cat/oHA. Powder in blended and pressed type, and hydrogel after hydration. (C) SEM images for powder(before hydration) and hydrogel(after hydration) of CHI-cat/oHA in various weight ratio(CHI-cat/oHA : 0.5, 1.0 and 2.0).

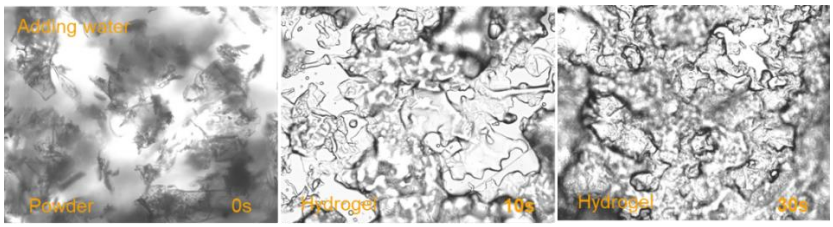
A


Powder state
Schiff base reaction(<5s)
Hydrogel
(crosslinked via imine bond)
oHA
CHI-cat
Aldehyde(-CHO)
Imine bond
Primary Amine
(-NH2)

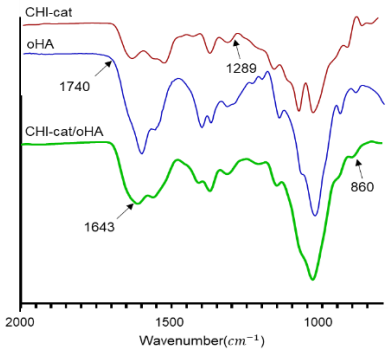
B


CHI-cat/oHA ratio
0.5
1.0
2.0
10mm

C


Adding water
Powder
0s
Hydrogel
10s
Hydrogel
30s

D


CHI-cat
oHA
CHI-cat/oHA
1740
1289
1643
860
2000
1500
1000
Wavenumber(cm⁻¹)

E

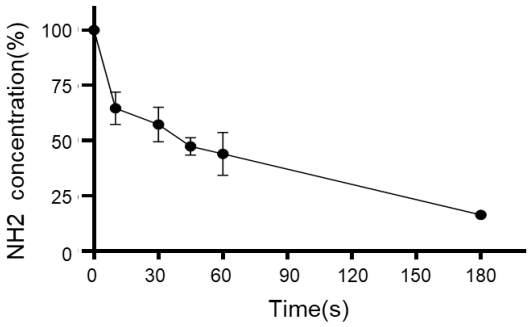
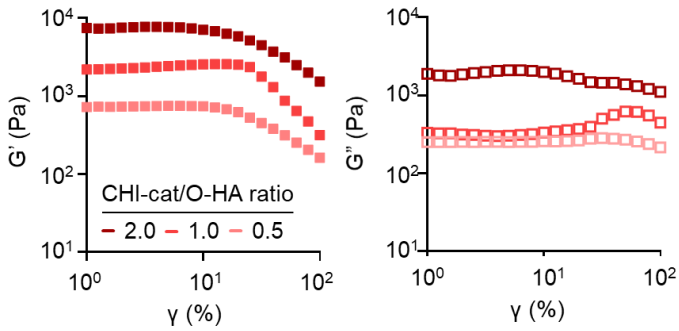


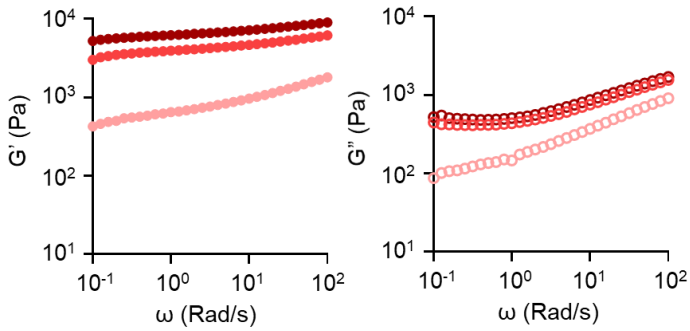
Figure 2.6 Rapid self-gelling of the powder via Schiff base reaction

(A) scheme illustration for the mechanism of fast gelling via Schiff base reaction (B) Sol-gel transition of CHI-cat/oHA hemostatic powder in various ratios via vial inverting test. (C) photographs of powder before and after hydration by using a microscope. (D) FTIR spectra of imine bond via Schiff base reaction between CHI-cat(amine group) and oHA(aldehyde group) (E) gelation kinetics by TNBSA assay (n=3)

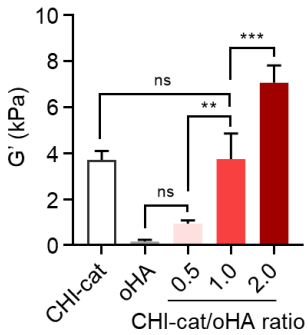
A


G' (Pa)
G'' (Pa)
γ (%)
CHI-cat/O-HA ratio
2.0
1.0
0.5

B


G' (Pa)
G'' (Pa)
ω (Rad/s)

C


G' (kPa)
ns
**
***
ns
CHI-cat
oHA
0.5
1.0
2.0
CHI-cat/oHA ratio

D

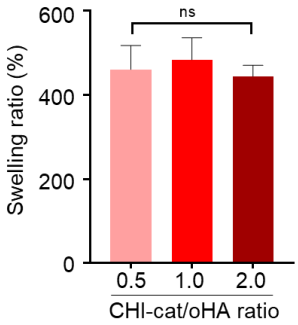
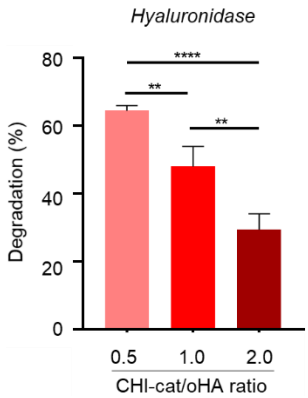


Figure 2.7 Mechanical property of the hemostatic powder

Rheological data for CHI-cat/oHA powder in various ratio (A) amplitude sweep test (B) frequency sweep test (C) storage modulus of each powder and (D) swelling ratio for CHI-cat/oHA powder in various weight ratio (n=3)

A



B

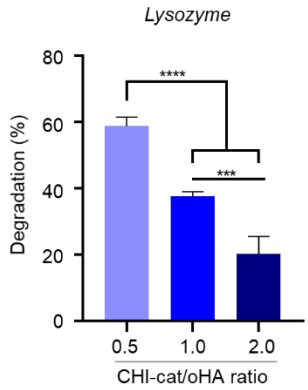


Figure 2.8 Degradability of the powder

Degradability of the powder after treating (A) 0.1U hyaluronidase and (B) lysozyme(1mg/ml) for 24 hours. (n=3)

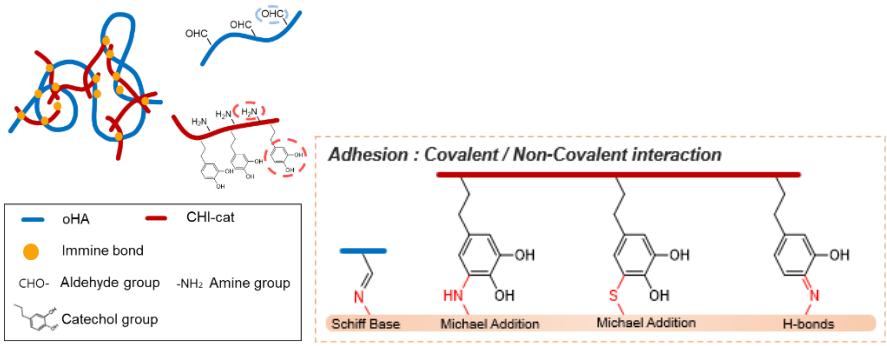


Figure 2.9 Wet adhesiveness via multiple functional groups

Scheme illustration for the mechanism of tissue adhesion of CHI-cat/oHA powder. Covalent and non-covalent interaction between functional groups of tissue and hemostatic powder

A

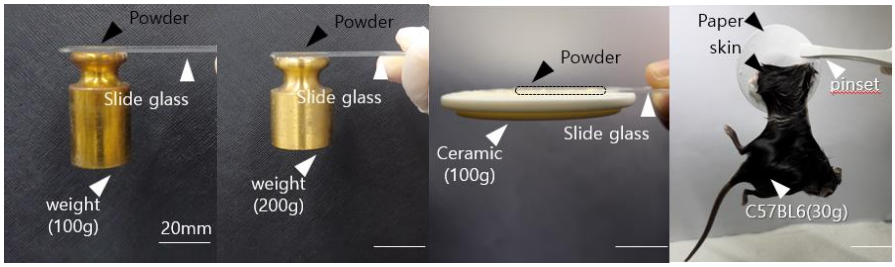
B

Figure 2.10 Robust bio adhesion of the powder

(A) Adhesion of CHI-cat/oHA powder fixed on a glass plate onto wet porcine skin in a short time(<2s)

(B) Adhesion of CHI-cat/oHA powder on wet porcine skin after 10, 30min

A



B

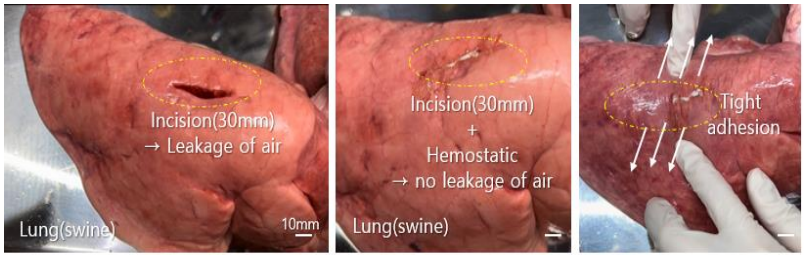
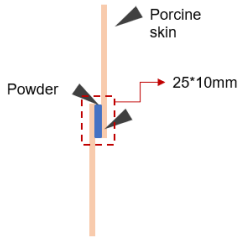


Figure 2.11 Robust adhesion of powder on various surfaces

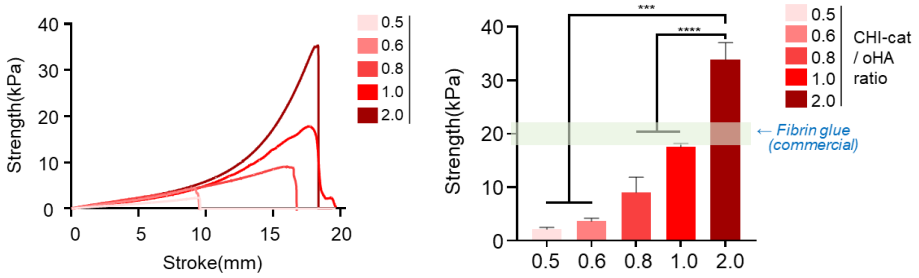
(A) Adhesion property of CHI-cat/oHA powder on various surfaces(brass, ceramic, glass, tissue etc)

(B) Wound sealing capacity of powder by swine lung(lobe) incision

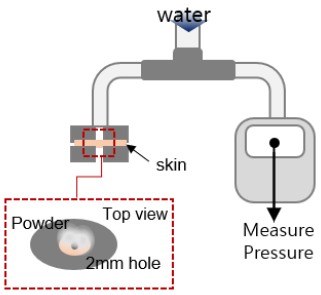
A



B



C



D

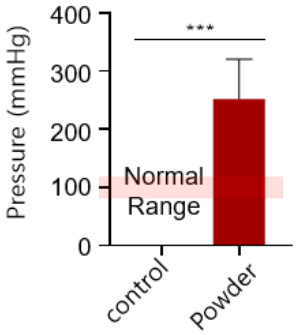


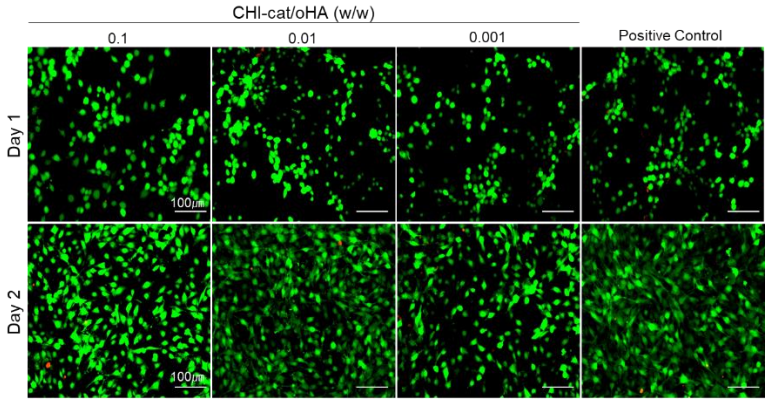
Figure 2.12 Adhesion strength of the powder

(A) scheme illustration for Lap shear test(ASTM F 2255)

(B) the adhesion strength of CHI-cat/oHA powder by lap shear test (n>3) (C) scheme illustration for bursting pressure test

(D) bursting pressure of CHI-cat/oHA(2.0) powder (n=3)

A



B

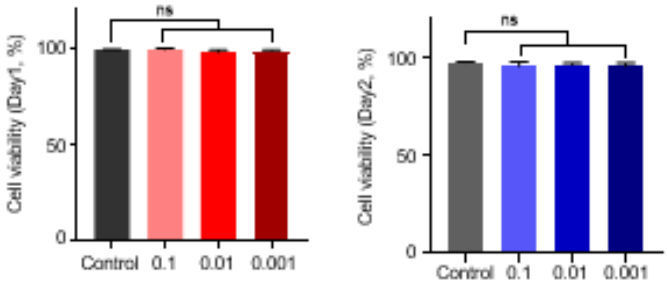


Figure 2.13 Biocompatibility of the powder

(A) Images of Live/Dead staining for day1, and 2 in different dilution ratios of CHI-cat/O-HA powder in growth media (positive control : growth media only, cell : NIT/3T3) (B) quantification of cell viability (%, n=3)

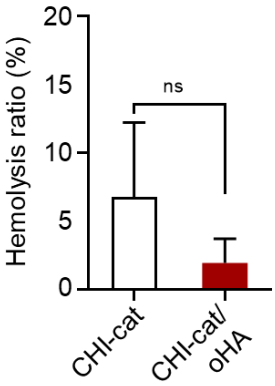


Figure 2.14 Hemocompatibility of the powder

quantification for hemolysis of each powder. PBS and Trion-X100 0.1% were used as control. (n=3)

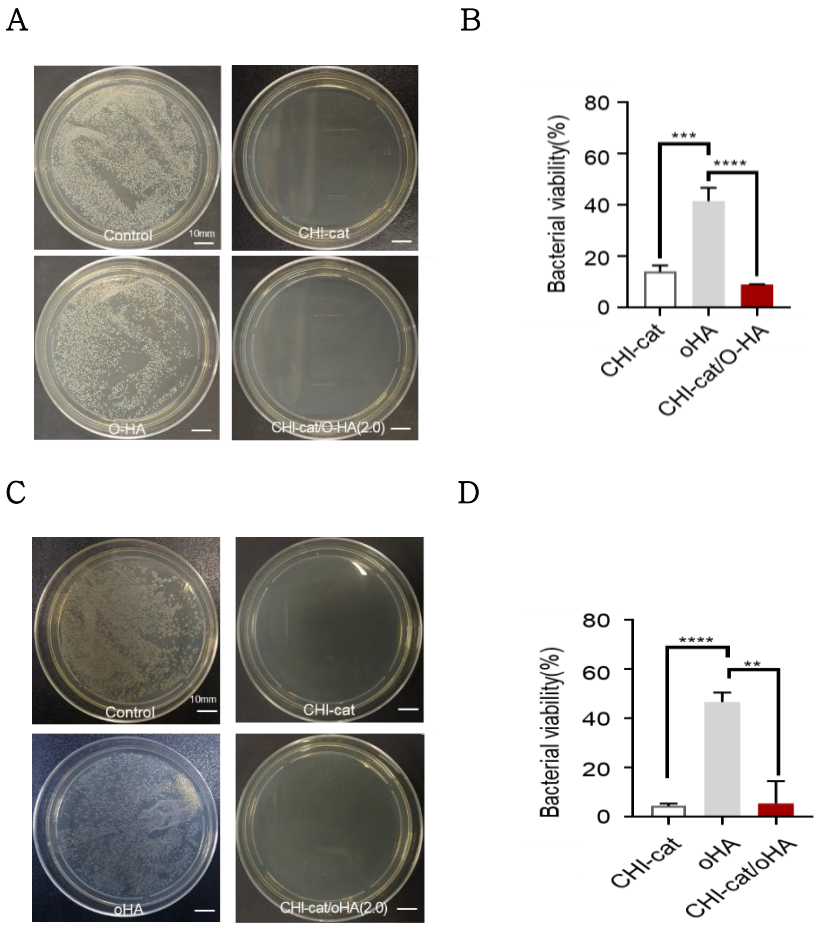
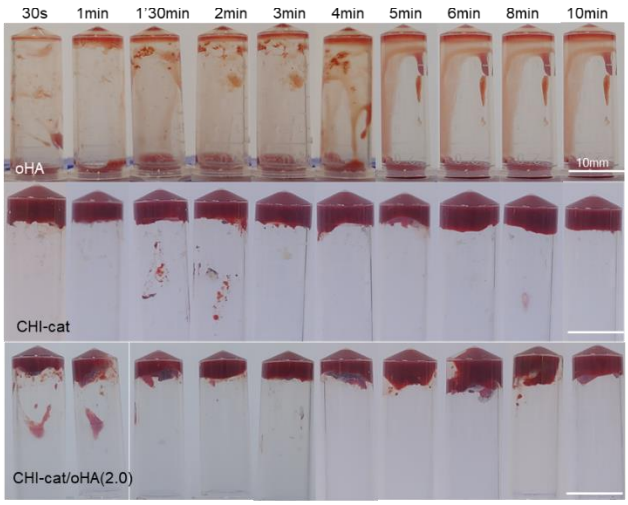


Figure 2.15 Antibacterial property of the powder

(A) Images of E.coli clones on agar plate derived from the survived E.coli after incubation with different powder at 37℃ for 12h (B) viability of E.coli after incubation with different powder at 37℃ for 12h (n=3) (C) Images of S.aureus clones on agar plate derived from the survived E.coli after incubation with different powder at 37℃ for 12h (D) viability of S.aureus after incubation with different powder at 37℃ for 12h (n=3)

A



B

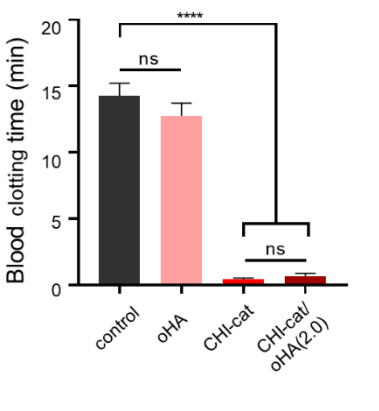
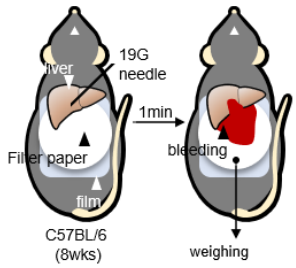


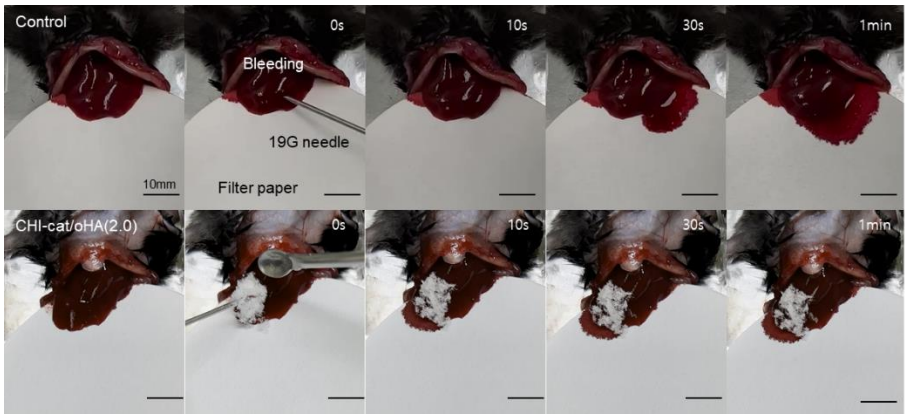
Figure 2.16 Blood coagulation test (in vitro)

(A) Photographs for blood coagulation test in vitro (B) quantification of blood clotting time of each powder(control : no treatment) (n=3)

A


liver
19G
needle
1min
Filter paper
bleeding
film
C57BL/6
(8wks)
weighing

B


Control
0s
10s
30s
1min
Bleeding
19G needle
10mm
Filter paper
CHI-cat/oHA(2.0)
0s
10s
30s
1min

C

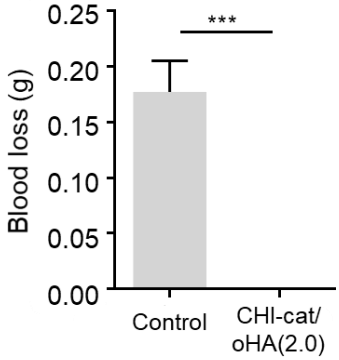
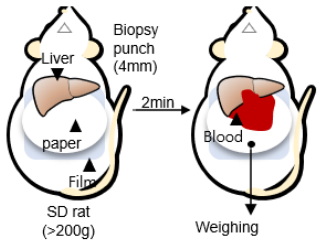

0.25
0.20
0.15
0.10
0.05
0.00
Blood loss (g)
***
Control
CHI-cat/
oHA(2.0)

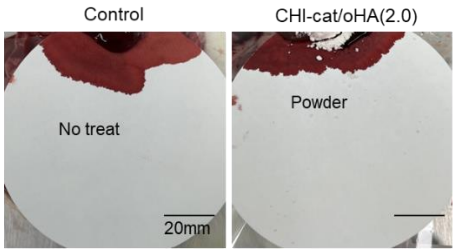
Figure 2.17 Hemostasis of adhesive powder (in vivo, mouse model)

(A) Scheme illustration for mouse liver bleeding model (B) Images of bleeding and hemostasis after applying CHI-cat/oHA powder (control : no treatment after bleeding) (C) quantification of lost blood from bleeding liver after treating powder (control : no treatment after bleeding) (n=3)

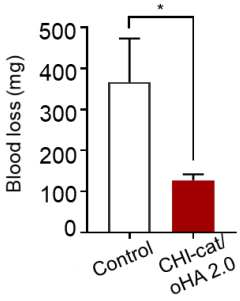
A


Liver
Biopsy
punch
(4mm)
2min
paper
Film
SD rat
(>200g)
Blood
Weighing

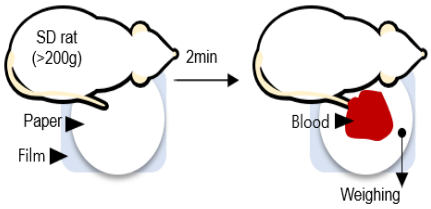
B


Control
CHI-cat/oHA(2.0)
No treat
Powder
20mm

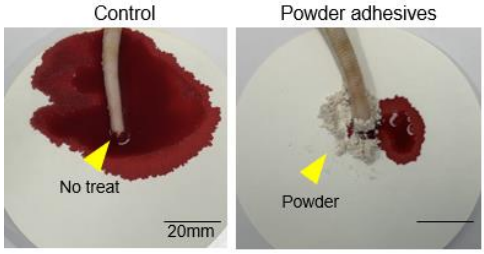
C


*
Blood loss (mg)
500
400
300
200
100
0
Control
CHI-cat/
oHA 2.0

D


SD rat
(>200g)
2min
Paper
Film
Blood
Weighing

E


Control
Powder adhesives
No treat
Powder
20mm

F

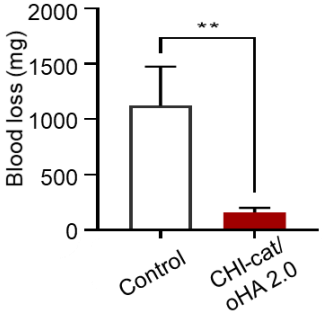
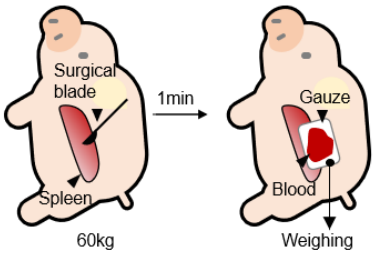

**
Blood loss (mg)
2000
1500
1000
500
0
Control
CHI-cat/
oHA 2.0

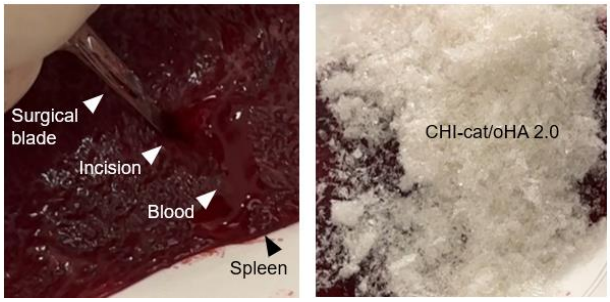
Figure 2.18 Hemostasis of adhesive powder (in vivo, rat model)

(A) Scheme illustration for rat liver bleeding model (B) images of liver bleeding and hemostasis after treating CHI-cat/oHA powder, no treatment after bleeding was used as control (C) quantification for loss of blood from liver bleeding model (D) scheme illustration for rat tail vein bleeding model (E) images of tail vein bleeding and hemostasis after treating CHI-cat/oHA powder, no treat after bleeding was used as control (F) quantification of loss of blood form tail vein bleeding model (n=3)

A



B



C

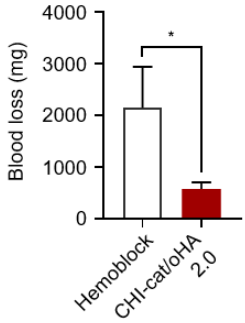
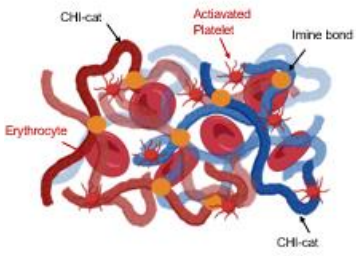


Figure 2.19 Hemostasis of adhesive powder (in vivo, porcine model)

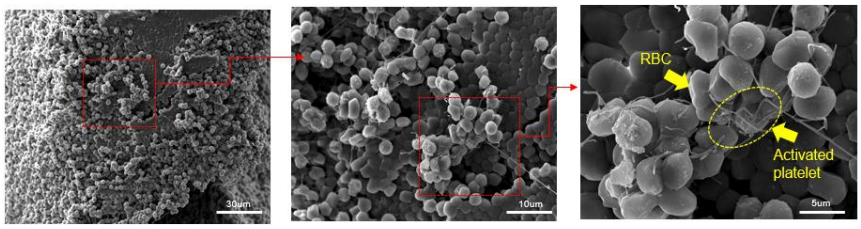
(A) Scheme illustration of porcine spleen bleeding model (B) images of spleen bleeding and hemostasis after treating CHI-cat/oHA powder, Hemoblock(chitosan-based, used in military) treat after bleeding was used as control (n=3)

A



B

C



D

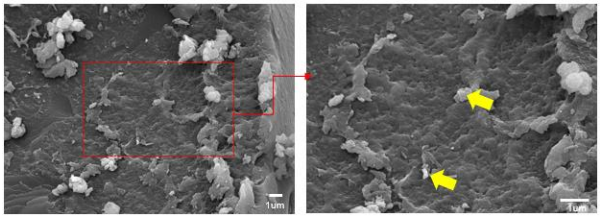


Figure 2.20 Mechanism of hemostasis

(A) scheme illustration for the mechanism of hemostasis of CHI-cat/oHA powder (B) images for the formation of blood protein barrier membrane by CHI-cat. oHA was dissolved in the blood. SEM images for (C) RBC adhesion on the surface of CHI-cat/oHA powder after treating whole blood (D) platelet adhesion on the surface of CHI-cat/oHA powder after treating PRP(platelet -rich plasma)

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초록(국문요약)

출혈은 전투상황에서 예방가능한 사망의 주요 원인으로 높은 비중을 차지하고 있다. 뿐만 아니라 매년 전세계적으로 100 만명 이상이 출혈로 인해 사망하고 있다. 출혈을 신속하게 처치하지 못할 경우 심각하게는 사망에 이를 수 있으며, 생존하더라도 장시간 조직으로의 산소 공급이 제한되어 장기 부전 등 심각한 후유증을 유발할 수 있다. 따라서 현장응급처치 단계에서의 신속한 지혈이 무엇보다도 중요하다.

본 연구에서는 카테콜-키토산과 산화히알루론산을 기반으로 한 분말형 지혈제를 개발하였다. 키토산의 아민기와 산화히알루론산의 알데하이드기에 의한 시프염기 반응에 의해서 빠르게 겔화되며, 뿐만 아니라 카테콜기 등 다양한 작용기와 조직과의 상호작용을 통해서 습윤한 출혈부위에 안정적으로 부착할 수 있다. 또한 키토산과 결합된 카테콜에 의해서 혈장 단백질과 막을 형성하고, 적혈구와 혈소판을 끌어당기고 활성화 시켜서 지혈과정을 촉진하게 된다. 이러한 지혈효과는 다양한 동물모델을 통해서 입증하였다.

카테콜-키토산과 산화히알루론산 기반의 분말 지혈제는 재난 및 응급상황에서 신속한 지혈을 통해 생존율을 향상시킬 수 있을 것으로 기대된다.

주요어 : 지혈, 신속한 겔화, 습식접착, 생체접학성, 향균성