Thesis for the Degree of Master of Engineering

Fabrication, Characterization and Biological Effects of Polyvinyl Alcohol Hydrogel Containing Chitooligosaccharides Conjugated with Gallic Acid

by

Hyeon-Ho Park

Interdisciplinary Program of Biomedical Mechanical & Electrical Engineering

Pukyong National University

February 23, 2018

Fabrication, Characterization and Biological Effects of Polyvinyl Alcohol Hydrogel Containing Chitooligosaccharides Conjugated with Gallic Acid

갈산-키토올리고당 함유 PVA 하이드로겔 제작과 특성 및 생물학적 효능 연구

Advisor: Prof. Won-Kyo Jung

by

Hyeon-Ho Park

A thesis submitted in partial fulfillment of the requirement

for the degree of Master of Engineering

in Interdisciplinary Program of Biomedical Mechanical & Electrical Engineering

Pukyong National University

February 2018

Fabrication, Characterization and Biological Effects of Polyvinyl Alcohol Hydrogel Containing Chitooligosaccharides Conjugated with Gallic Acid

A dissertation

by

Hyeon-Ho Park

Approved by:

(Chairman) Young-Mog Kim Ph. D

(Member) Seung-Yun Nam Ph. D

(Member) Won-Kyo Jung Ph. D

February 23, 2018

Contents

List of tables

List of figures

List of abbreviations

^{1}H-NMR	Proton nuclear magnetic resonance
^{13}C-NMR	Carbon nuclear magnetic resonance
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
BHI	Brain heart infusion broth
CFU	Colony forming units
COS	Chitooligosaccharides
COS I	COS with molecular weight of 1-3 kDa
COS II	COS with molecular weight of 3-5 kDa
COS III	COS with molecular weight of 5-10 kDa
COS/PVA	5 wt% PVA hydrogel with 1 wt% COS III
D_2O	Deuterium oxide
DCFH-DA	5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate
DMEM	Dulbecco's minimum Eagle's medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Deionized water
EDTA	Trypsin/ethylenediaminetetraacetic acid
FBS	Fetal bovine serum

G′	Storage modulus
GA	Gallic acid
GA-COS	COS combining with GA
GA-COS I	COS with molecular weight of 1-3 kD combining with GA
GA-COS II	COS with molecular weight of 3-5 kD combining with GA
GA-COS III	COS with molecular weight of 5-10 kD combining with GA
GA-COS/PVA	5 wt% PVA hydrogel with 1 wt% GA-COS III
GSH-Px	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HaCaT	Human keratinocyte
IC_{50}	50% inhibition concentration
ISO	International Organization for Standardization
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MIC	Minimum inhibitory concentration
NHDF	Normal human dermal fibroblast

P. acnes	Propionibacterium acnes
PBS	Phosphate buffer saline
PVA	Poly(vinyl alcohol)
PPVA	Pure 5 wt% PVA hydrogel
RAW 264.7	Mouse macrophage
ROS	Reactive oxygen species
SD	Standard deviation
SOD	Superoxide dismutase
W_o	Initial weight of dried hydrogel
W_e	Weight of re-dried hydrogel after soaking

Fabrication, Characterization and Biological Effects of Polyvinyl Alcohol Hydrogel Containing Chitooligosaccharides Conjugated with Gallic Acid

Hyeon-Ho Park

Interdisciplinary Program of Biomedical Mechanical & Electrical Engineering

College of Engineering

Pukyoung National University

Abstract

여드름은 생명에 직접적으로 위협을 주는 질병은 아니지만, 외모를 중시하는 현대사회에서 매우 주목받는 질병 중 하나이다. 여드름은 청소년뿐만 아니라 성인에게서도 염증을 유발하여 흉터, 색소침착 등 후유증을 남기는 만성적인 피부질환이다. 여드름은 흡연, 음주, 호르몬 변화, 스트레스 등 많은요인에 의해 생성되는 과도한 피지가 *Propionibacterium acnes* (*P.acnes*)을 증식할 수 있는 환경을 만들어 준다. 그리고 균에 감염된 모낭은 염증 유발과 피부자극이 일어나며 여드름으로 발전된다. 게다가 *P.acnes* 는 활성산소억제 효소 또한 억제시켜 과도하게 생성되는 활성산소에 의해 지속적인 피부자극과 여드름을 발생시킨다. 그로 인해 *P.acnes* 증식을 막기 위해 Macrolide, Tetracycline, Clindamycin, azelaic acid and erythromycin 등의 항생제들이 여드름치료를 위해 사용되고 있다. 하지만 이런 항생제들은 항생제 내성, 알러지, 소화 기능 저하 등 여러 부작용을 발생시킨다. 이에 본 연구에서는 천연물질들을 합성하여 여드름 치료제를 개발 및 적용하였다. 사용된 물질은 항균효과가 뛰어나다고 알려져 있는 천연 다당류 키토산을 1-3, 3-5, 5-10 kDa 으로 저분자화 시킨 키토올리고당과 항산화 효과가 뛰어난 갈산을 사용하였고 Hydrogen peroxide mediated method 로 갈산-키토올리고당을 합성하였다. 합성이 제대로 되었는지는 UV-vis 와 ^{1}H, ^{13}C-NMR spectra 로 평가하였고 항산화와 항균실험을 통해 여드름 치료제 활용 가능성을 확인하였다 (Fig. 7-9, Table 2-3).

그리고 *P.acnes* 에 가장 우수한 항균효과와 항산화 효과를 나타낸 갈산-키토올리고당과 키토올리고당 (5-10 kDa)을 합성고분자 Poly vinyl alcohol 에 혼합해 하이드로겔을 제작하였다. 제작된 하이드로겔을 실험한 결과, 적절한 물리적 강도를 가지면서 피부를 구성하는 섬유아세포, 각질세포 및 대식세포에 대한 독성을 지니지 않는 것을 확인할 수 있었다 (Fig. 10-13). 대식세포에 대한 H_2O_2 억제능을 실험한 결과, 갈산-키토올리고당을 함유한 하이드로겔만이 대식세포 내 H_2O_2 을 억제하는 것을 관찰할 수 있었다 (Fig. 14). 그리고 창상피복재의 항균실험으로 사용되는 ASTM E2149 실험결과, *P.acnes* 에 키토올리고당과 갈산-키토올리고당을 함유한 하이드로겔이 우수한 항균효과를 가지는 것을 확인할 수 있었다 (Fig. 15). 이러한 결과들을 토대로 갈산-키토올리고당 함유 하이드로겔은 모낭 내 *P.acnes* 증식을 억제하여 염증반응이 일어나는 것을 차단해줄 뿐만 아니라 과도하게 생성되는 활성산소로부터 피부조직을 보호해주어 여드름 치료에 효과적일 것으로 판단된다. 그리고 여드름 치료용 마스크 팩 혹은 패치로 활용이 가능할 것으로 사료된다.

1. Introduction

1.1. Acne vulgaris

Developing standard of living with industrialization, the desire of people for beautiful appearance have been increased. And, health care and cosmetic industry has rapidly grown to meet requirements (1). Among much things, the products and therapies associated with acne vulgaris are the most popular one, because acne vulgaris is one of the most common chronic skin disease resulting in disfiguration and scarring. According to recent studies, almost all teenage suffer from acne vulgaris and about 60% of those aged 20 to 29 years had acne vulgaris (2-4). The bacteria flourish is considered to be one of the main cause contributing to the development of acne vulgaris (Fig. 1) (5). In particular, *Propionibacterium acnes* (*P. acnes*) in sebaceous follicles plays a key cause contributing to acne vulgaris pathogenesis in that *P. acnes*, an anaerobic Gram-positive organism, results in more sebum production, excessive cytokines and chemotaxis responsible for inflammation in keratinocytes and macrophages (6). In addition, *P. acnes* decrease enzymes which may be involved in defense system against oxidative stress and form a biofilm within follicles leading to decreased response to antimicrobial agent (7). While neutrophils produce reactive oxygen species (ROS) for expelling of *P. acnes* without regulation of enzymes, excessive generation of ROS might continuously induce oxidative damage in normal cell constituents and clinical populopustular type acne vulgaris as well as accelerate a skin senescence (Fig. 2) (8-10). Antibiotics, such as the macrolides, tetracyclines, clindamycin, azelaic acid and erythromycin, are used to reduce *P. acnes* proliferation and inflammation. However, the antibiotic treatment increase bacteria resistance and is associated with side effects including cytotoxicity, allergic and diarrhea (7,11). So, resent studies have focused on the development of alternative antimicrobial materials with less side effects.

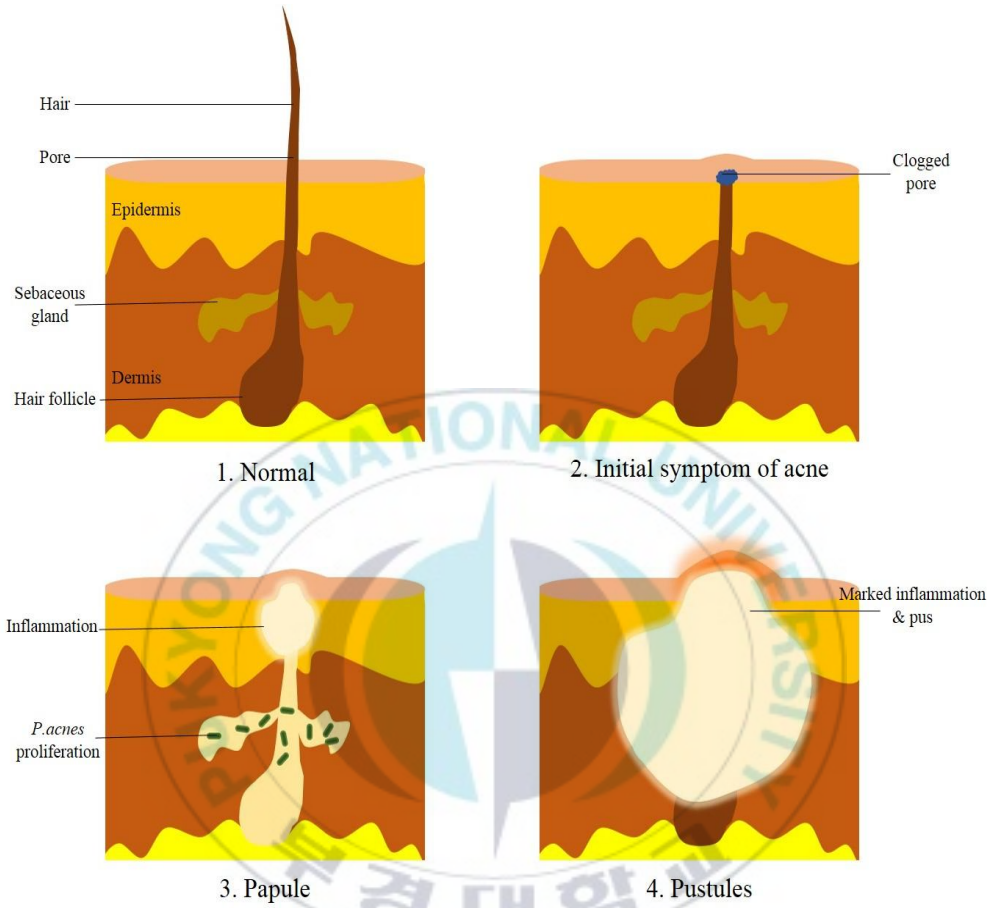


Fig. 1. Acne vulgaris occurrence process. There are four steps: (1) Normal, (2) Initial symptom, (3) Papule, (4) Pustules.

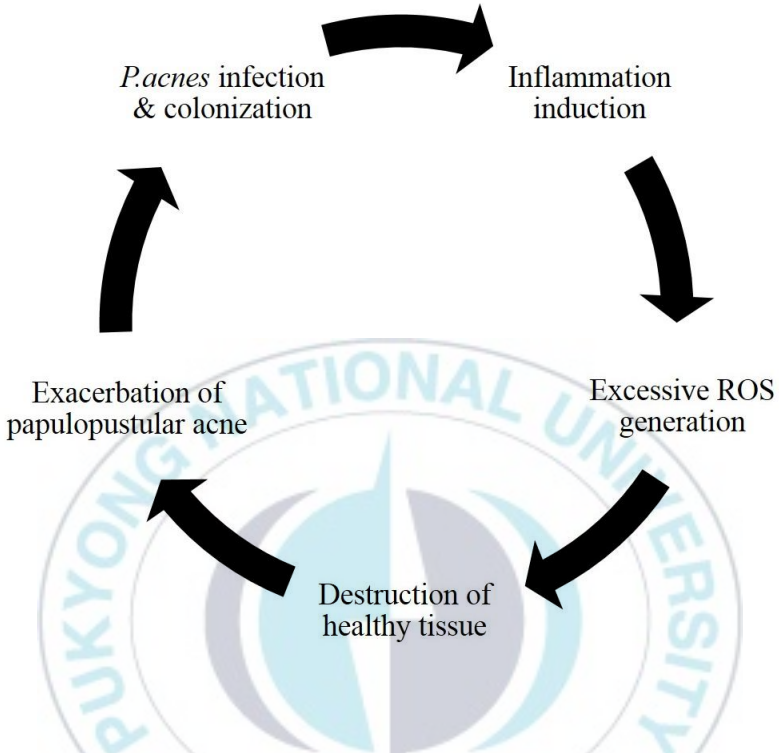


Fig. 2. The cycle explaining chronic skin disease by *P.acnes* infection and colonization.

1.2. Chitooligosaccharides (COS)

Chitosan, a partially deacetylated linear polysaccharide of N-acetyl glucosamine, is obtained from chitin, natural polysaccharide found in the exoskeleton of crustaceans, by deacetylation with a strong alkaline and was known as material having anti-cancerogenic (12), anti-diabetic (13), anti-angiogenic (14) and anti-microbial effects (15). Especially, chitosan has an excellent antimicrobial property against fungus, gram-positive and gram-negative bacteria without toxicity. For these reasons, chitosan has been applied in various fields. However, chitosan is limited practical application in a biomedical filed due to low solubility property in neutral pH conditions. COS is chitosan with molecular weight less than 10 kDa (Fig. 3) (16). COS are obtained by acid hydrolysis, physical and enzymatic degradation. Recently, COS have attracted much attention for pharmaceutical and medical applications, due to their high solubility property, biodegradability and biocompatibility (17). In addition, although it was previously known that the antibacterial activity of chitosan and COS is generally dependent on their molecular weight and the level of deacetylation, the antibacterial activity of COS against certain bacteria is more effective than chitosan in mechanisms of chitosan for anti-microbial activity (18,19). Furthermore, COS exhibit an anti-oxidant activity with various biological activities. However, 50% inhibition concentration (IC_{50}) values for ROS were observed at high concentration of COS treatment in the previous studies (20). That being so, synthesis of phenolic compounds and COS to enhance antioxidant activity and physiological property have been gained a great deal of interest.

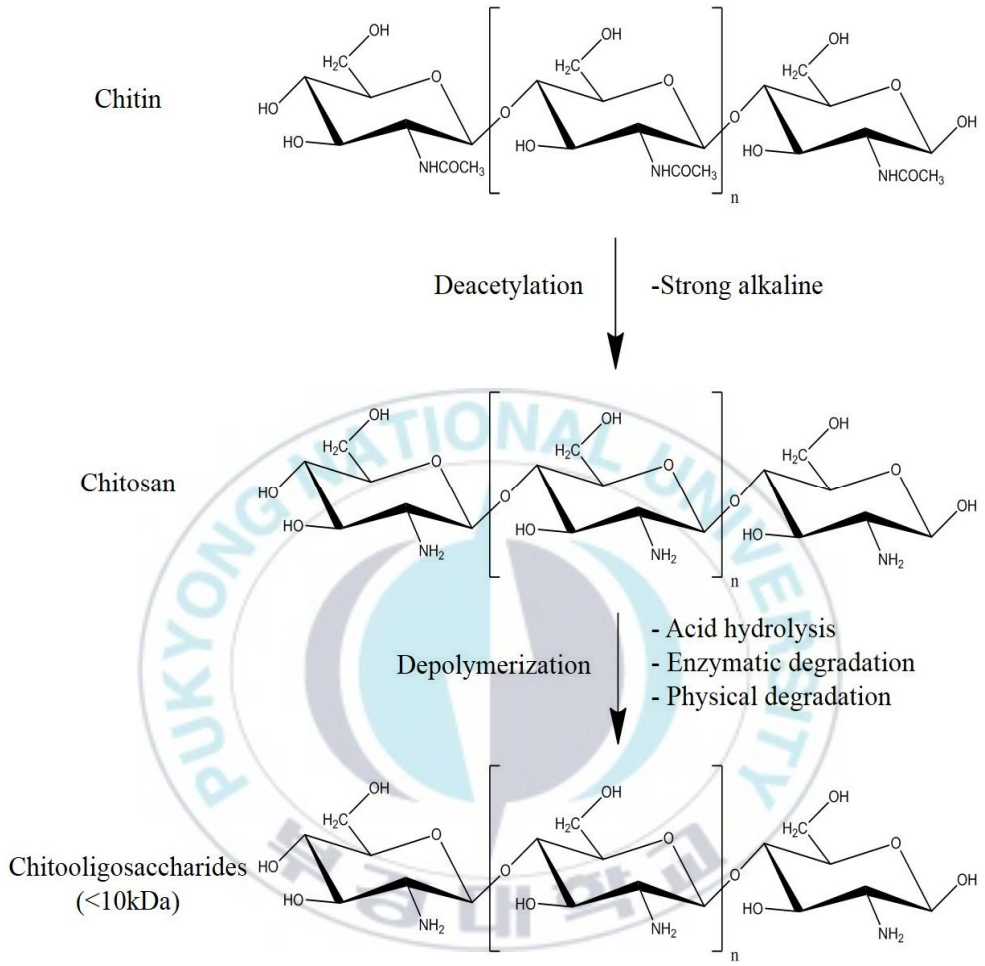


Fig. 3. Chemical structure of chitin, chitosan and chitooligosaccharides.

1.3. Gallic acid (GA)

GA (3,4,5-trihydroxy benzoic acid), an endogenous plant polyphenol, is one of abundant phenolic compounds in tea, grapes, berries and fruits. GA has been shown to possess diverse biological activities, such as anti-oxidant, anti-inflammatory and anticancer effects. These functions have leaded it to conjugate with COS by chemical method and COS combining with GA (GA-COS) have shown increased biological activities. For example, and GA-COS which were synthesized by mixing two solution (solution A: COS dissolved in methanol adjusted to pH 6.8 with triethylamine, solution B: GA dissolved methanol and dicycloexylcarbodiimide) showed greatly improved inhibitory effects on intracellular free radical generation and antigen-induced allergic reactions in RBL-2H3 mast cells (21,22).

1.4. Hydrogel

The hydrogel is basically a three-dimensional complex network containing a lot of water to maintain a moist environment at the wound site and allow gaseous exchange (23,24). It means that hydrogel can make effective wound healing conditions to provide a wet environment compared with a dry environment (25). For these advantages, the hydrogel not only has been developed as wound dressing but also has received considerable attention in recent cosmetic and medical industries (26,27).

1.5. Poly(vinyl alcohol) (PVA)

PVA is a linear synthetic polymer prepared by hydrolysis of poly vinyl acetate (Fig. 4A) (28). PVA, which have just hydroxyl groups, is commonly used in medical application due to its biocompatibility, high water solubility and nontoxicity. Furthermore, PVA is easily cross-linked to form hydrogel by repeated cycles of

freezing and thawing without requiring the use of toxic chemicals (Fig. 4B) (29,30). The inner network of PVA hydrogel formed by freezing and thawing method contains free water, crystalline PVA domains which act as knots of networks and swollen amorphous PVA which forms porous wall (31). This advantage has developed PVA hydrogels blended with bioactivity materials to enhance the cell adhesion, growth, proliferation and differentiation as well as anti-bacterial activity and mechanical property in recent studies (32,33).

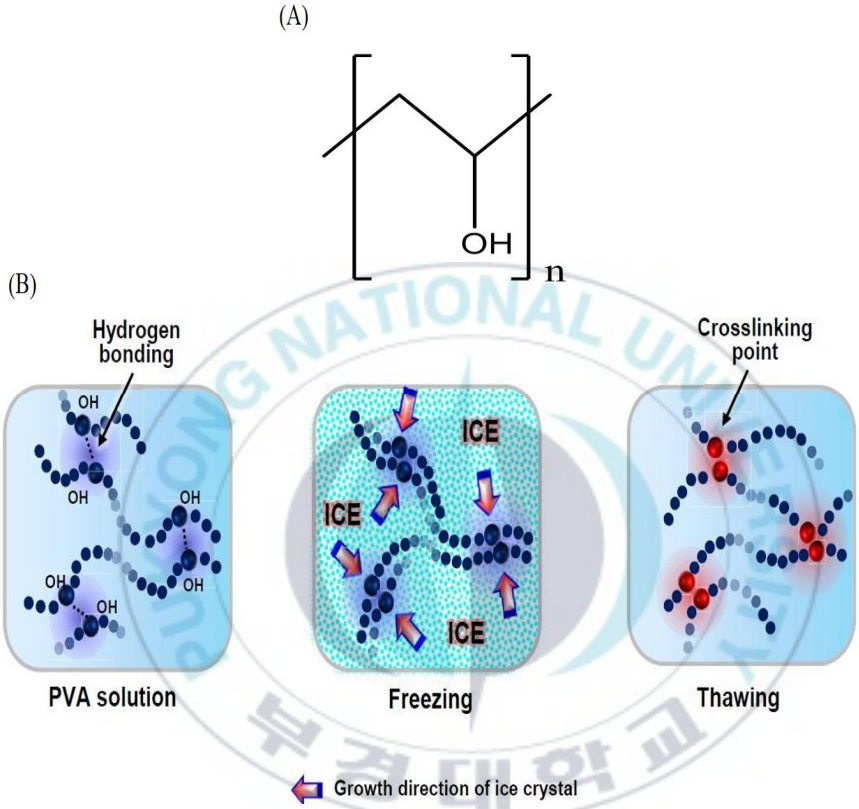


Fig. 4. Chemical structure of poly(vinyl alcohol) (PVA) (A) and diagram of PVA hydrogel formation by freezing thawing method (B) (Kim et al., 2015)

2. Materials and methods

2.1. Materials

COS (Molecular weight: 1-3, 3-5, 5-10 kDa; degree of deacetylation ≥80%) were purchased from Kitto Life Co. (Seoul, Korea). GA, PVA (Molecular weight: 89,000-98,000; 99+% hydrolyzed), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Hydrogen peroxide (H_2O_2), peroxidase, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (MTT) and deuterium oxide (D_2O) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Dulbecco's minimum Eagle's medium (DMEM), Fetal bovine serum (FBS), trypsin/ethylenediaminetetraacetic acid (EDTA) solution, antibiotic/antimycotic solution, phosphate buffer saline (PBS) and other materials used in cell culture experiment were purchased from GIBCO™ (Gaithersburg, MD, USA). All other chemicals and solvents were of analytical grade, and water used in experiment was deionized.

2.2. Preparation of GA-COS

The GA-COS were synthesized by the hydrogen peroxide mediated method with some modification (Fig. 5) (34,35). Briefly, 1 g of each COS with molecular weight of 1-3, 3-5 and 5-10 kDa (COS I, COS II and COS III) were dissolved in 100 mL of deionized water (DW) and 2 mL of 1.0 M H_2O_2 containing 0.108g of GA was added. After 30 min at room temperature, the amount of GA with 0.5 molar ratio to COS were added to the mixture and the reaction was carried out for 24 h. The resulting solution was dialyzed by dialysis tube (MWCO 1000 Da) 48 h and lyophilized. GA-COS with molecular weight of 1-3, 3-5 and 5-10 kDa were respectively designated as GA-COS I, GA-COS II and GA-COS III.

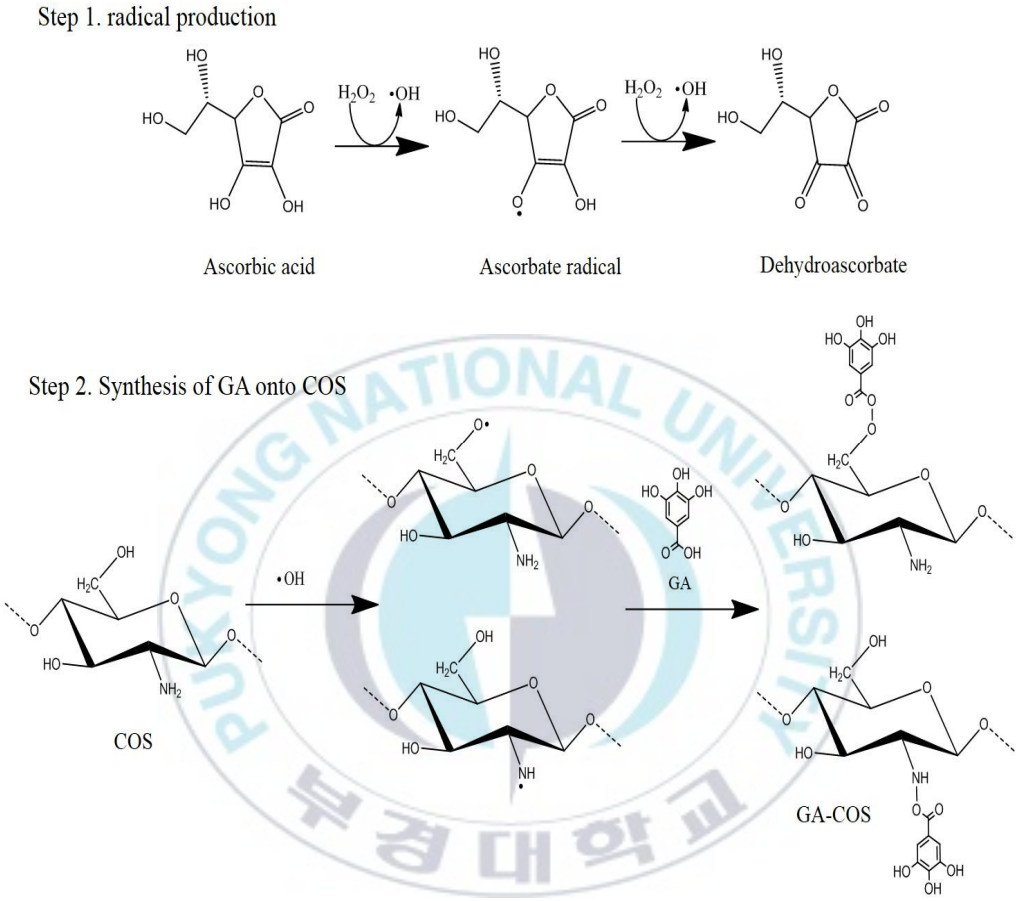


Fig. 5. Synthesis mechanism by the hydrogen peroxide mediated method; COS: chitooligosaccharides, GA: gallic acid, GA-COS: COS combining with GA.

2.3. Characterizations of GA-COS

2.3.1. Analytical determinations of GA-COS

The UV-vis spectra were determined by a UV-vis microplate reader (BioTek Instruments, Winooski, VT, USA) by scanning from 200 to 600 nm.

Samples were dissolved in D_2O. Then the proton and carbon nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) spectra for samples were recorded by a JEOL JNM ECP-600 FT-NMR spectrometer (Japan) under a static magnetic field of 600 MHz.

2.3.2. Determination of grafting degree in GA-COS

The GA content in GA-COS was measured by the Folin-Ciocalteu method with some modification (36). Briefly, 20 μL of all GA-COS (1mg/mL) was mixed with 100 μL of Folin-Ciocalteau reagent and followed by the addition of 80 μL of 7.5% Na_2CO_3. The mixture was shaken and maintained under room temperature for 30 min, and the absorbance of the mixture was read at 750 nm using a UV-vis microplate reader. GA was used as a standard.

2.4. Anti-oxidant activity of GA-COS

2.4.1. DPPH radical scavenging assay

The DPPH radical scavenging activity was assayed according to the previous method with slight modification (37). Samples (COS I, II, III, GA-COS I, II and III) were dissolved in DW for preparation of a series of concentrations to obtain IC_{50} value. Then, 100 μL of samples mixed with 100 μL in 96-well microplate. After incubation at room temperature for 30 min. the absorbance of mixture was measured at 517 nm by a UV-vis microplate reader.

2.4.2. ABTS radical scavenging assay

The spectrophotometric analysis of ABTS radical scavenging activity was performed following a previous report with modification. Briefly, 7 mM ABTS solution was reacted with 2.45 mM ammonium persulfate and the mixture incubated in the dark for 12 h to generate ABTS radicals. Then, a mixture was diluted with DW until the value of absorbance at 745 nm was reached to 0.7 ± 0.02. 150 μL of ABTS radical solution was mixed with 50 μL of samples in 96-well microplate and the absorbance of mixture was monitored at 745 nm.

2.4.3. H_2O_2 scavenging assay

The H_2O_2 scavenging assay was performed using a previously described procedure. 100 μL of 0.1M phosphate buffer and samples were mixed in a 96-well microplate. After incubation with 20 μL of H_2O_2 for 5 min at 37°C, mixture was reacted with 30 μL of 1.25 mM ABTS and peroxidase (1 unit/mL) for 10 min at 37°C. The absorbance was read at 405 nm.

2.5. Anti-bacterial activity of GA-COS

2.5.1. Strains and culture conditions

P. acnes was obtained from the Korean Collection for Type Cultures (Daejeon, Korea); *P. acnes* KCTC 3314. *P. acnes* strains were anaerobically cultivated in brain heart infusion broth (BHI) supplemented with 1.0% dextrose and incubated at 37°C for 72 h in a CO_2 incubator. The used samples were dissolved in 1 x 10^{-5} N HCl (pH 5).

2.5.2. Measurement of minimum inhibitory concentration (MIC)

MIC assay was performed following guideline of Clinical and Laboratory Standards Institute (CLSI, 2012). MIC values were determined as the lowest concentration which

inhibits visible growth of microorganisms, after *P. acnes* strains were inoculated with samples into BHI for 24 h at 37°C.

2.5.3. Disk diffusion assay

The anti-bacterial activity of COS and GA-COS against *P. acnes* was carried out by disk diffusion assay. *P. acnes* strains were spread on blood agar-based plate with paper discs (6 mm in diameter) containing 5 mg of samples. After incubating for 24 h at 37°C, the diameter of the growth inhibition zone was measured by Vernier calipers.

2.6. Fabrication of hydrogels

PVA hydrogels blended with COS III and GA-COS III were fabricated by a freezing-thawing method. Aqueous 10 wt% PVA solution was prepared by dissolving PVA into DW in the water bath at 70 °C for 2 h followed by cooling at room temperature for 1 h. 2 wt% COS III and GA-COS III solutions were prepared, dissolved in 2 x 10^{-5} N HCl. PVA solution was mixed at a ratio of 1:1 with COS III and GA-COS III solutions. After mixing, each mixture was poured into 24-well plates with a volume of 0.5 ml per well and underwent repeated freezing-thawing cycles three times, consisting of 18 h freezing at -80°C and 6 h thawing steps at room temperature. Consequently, a pure 5 wt% PVA hydrogel (PPVA), 5 wt% PVA hydrogel with 1 wt% COS III (COS/PVA), and 5 wt% PVA hydrogel with 1 wt% GA-COS III (GA-COS/PVA) were fabricated (Fig. 6).

2.7. Characterizations of hydrogels

2.7.1. Water swelling analysis

Dried hydrogels (PPVA, COS/PVA, GA-COS/PVA) was initially weighed and immersed in DW at 37°C until reaching the equilibrium state for water-uptake

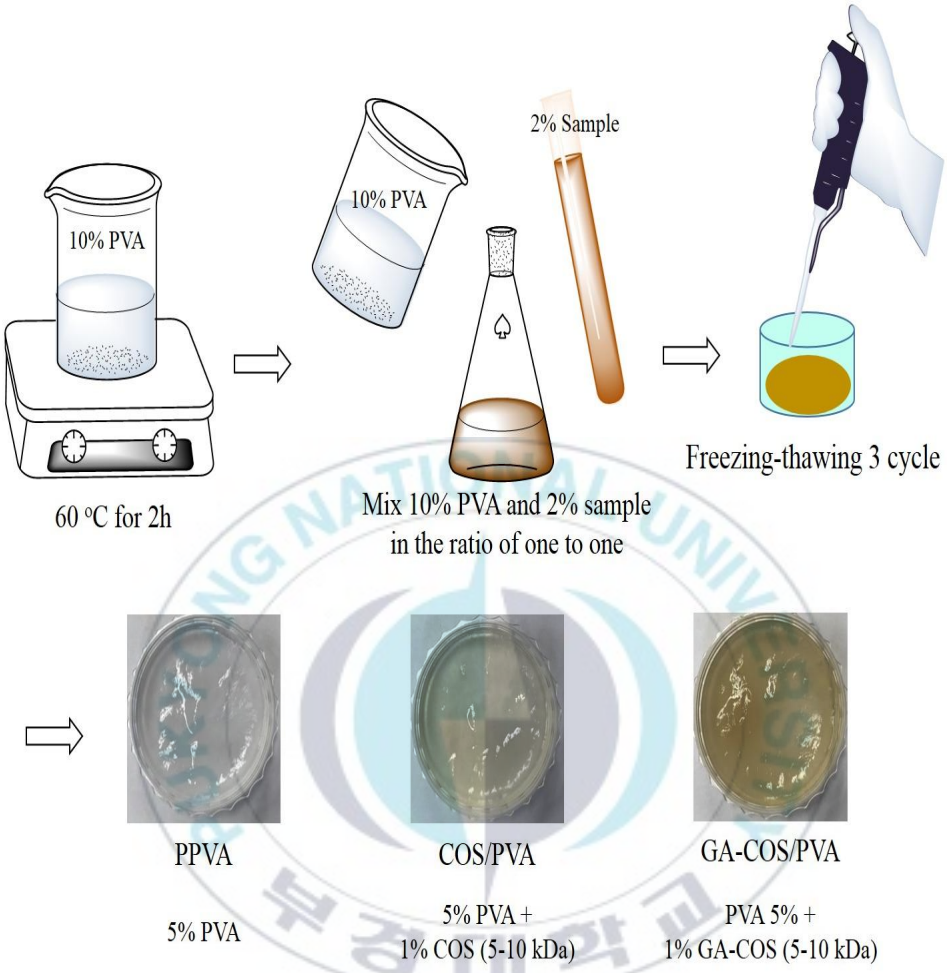


Fig. 6. The schematic fabrication process of PPVA, COS/PVA and GA-COS/PVA

measurements. The dry weight (W_d) of the hydrogels was determined by lyophilizing, and the swollen weight (W_s) of the hydrogels was determined by blotting the surface water with blotting paper. The swelling ratio of hydrogels was calculated using the following formula:

$$\text{Swelling ratio } (\%) = \left[\frac{(Ws-Wd)}{Wd}\right] \times 100 \ \%$$

2.7.2. Gel fraction analysis

The hydrogels were prepared by dried at 50°C under vacuum and weighted (W_o). The dried hydrogels were soaked in DW for 24 h. Then, the hydrogels were dried again to remove the soluble parts and hydrogels dried after soaking were weighted (W_e). The gel fraction percentage was determined as follows equation:

$$\text{Gel fraction } (\%) = \left[\frac{We}{Wo}\right] \times 100 \ \%$$

2.7.3. Rheological properties

The dynamic mechanical analysis was performed by Discovery HR-2 Hybrid Rheometer with 20 mm parallel plate geometry (TA Instruments, USA). Hydrogels loaded with a static load of 0.05 N at 25°C and deformed at constant amplitude over a range of frequencies (0.1 Hz - 10 Hz) to measure storage modulus (G').

2.8. Cell experiments

2.8.1. Cell culture

Cell experiments were performed using normal human dermal fibroblast (NHDF), human keratinocyte (HaCaT) and mouse macrophage (RAW 264.7). Cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic

solution in an incubator with a humidified atmosphere of 5% CO_2 at 37°C. Cells were cultured until reaching 70–80% confluence. NHDF and HaCaT were detached by the addition of trypsin/EDTA solution, while RAW 264.7 was detached by scraper. Cells were counted using a hemocytometer and were seeded into cell culture plates.

2.8.2. Cytotoxicity test of hydrogels

The cytotoxicity of hydrogels was evaluated following International Organization for Standardization (ISO) 10993-5 with some modifications (38,39). To obtain extract medium, hydrogels sterilized by UV irradiation for 1 h were immersed in culture medium for 24 h at 37°C. Cells were seeded in 96 well plates at a density of 1 x 10^4 (NHDF and HaCaT) and 1 x 10^5 cells/wall (RAW 264.7) and incubated with extract medium and flesh medium (control) for 24 h. Cell viability was determined by the alamar blue assay (40).

2.8.3. Measurement of intracellular ROS

The production of intracellular ROS was detected by oxidation of the cell permeable fluorescence dye, 5-(and-6)-carboxy-2′,7′-dichlorofluorescin diacetate (DCFH-DA, Sigma-Aldrich) (41). Each extract medium was prepared by incubation with hydrogels for 1 h. Twenty-four hours prior to injection of extract mediums, RAW 264.7 was seeded on 24-well plate at concentration of 5 x 10^5 cells/wall. Each extract mediums and flesh medium (blank and control) were treated for 1 h, and then adding 0.5 mM H_2O_2 except blank, cells were incubated for 30 min. After that, cells were respectively incubated with 20 μM DCFH-DA and 6 μg/mL DAPI for 30 min. the images were acquired by a fluorescence microscope (Zeiss, Germany).

2.9. Anti-bacterial activity of hydrogels

The anti-microbial activity of hydrogels against *P. acne* was performed by ASTM E2149-01, standard methods for determining the antimicrobial activity of immobilized antimicrobial agent under dynamic contact conditions (42,43). Briefly, hydrogels were sterilized by UV irradiation and immersed in a solution with *P. acne* suspension (10^5 CFU/mL) at 37°C. Aliquots were taken from the solution at various times in order to assess a viable cell-counting method. The surviving microorganisms were counted in triplicate as colony forming units (CFU).

2.10. Statistical analysis

All quantitative data are presented as means ± standard deviation (SD), with at least three individual experiments conducted using fresh reagents. Significant differences among each group were assessed using one-way analysis of variance (ANOVA) followed by Duncan's test using the statistical software PASW Statistics 21.0 (SPSS Inc.). The differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Characterizations of GA-COS

3.1.1. Structure analysis

The UV-vis spectra for GA, all COS and GA-COS is observed in Fig. 7. The absorbance peaks of GA-COS I, II and III showed that COS I, II and III were well synthesized with GA by the hydrogen peroxide mediated method. COS did not have any absorbance peak in the range 200 to 600 nm, while all GA-COS had two absorbance peaks at 212 and 262 nm. It is identical to the characteristic absorbance peaks of GA, which should be assigned to the π-system of benzene ring (44).

The successful synthesis was confirmed by ^{1}H-NMR and ^{13}C-NMR analysis. As shown in Fig. 8, resonance peaks of all GA-COS occurred at 2.04, 3.09, 3.32-3.91, 4.53, 4.80, 5.41 and 7.03 ppm attributed to H-Ac, H_2, H_3-H_6, β-H_1, D_2O, α-H_2 and GA, respectively, whereas a resonance peak correspond to protons of GA did not appeared in resonance peaks of COS. In addition, GA-COS III showed characteristic resonance peaks of aromatic carbon at 109.43, 127.75, 135.75 and 144.34 ppm (Fig. 9) (45,46).

3.1.2. Measurement of GA contents in GA-COS

The antioxidant activity of synthetic product depends on the amount of grafted antioxidant molecules on the backbone of polymers and was predicted by Folin-Ciocalteu method. GA contents in GA-COS I, II and III were rarely different, showing 80.95, 88.58 and 85.35 mg GA/g GA-COS, respectively (Table 1).

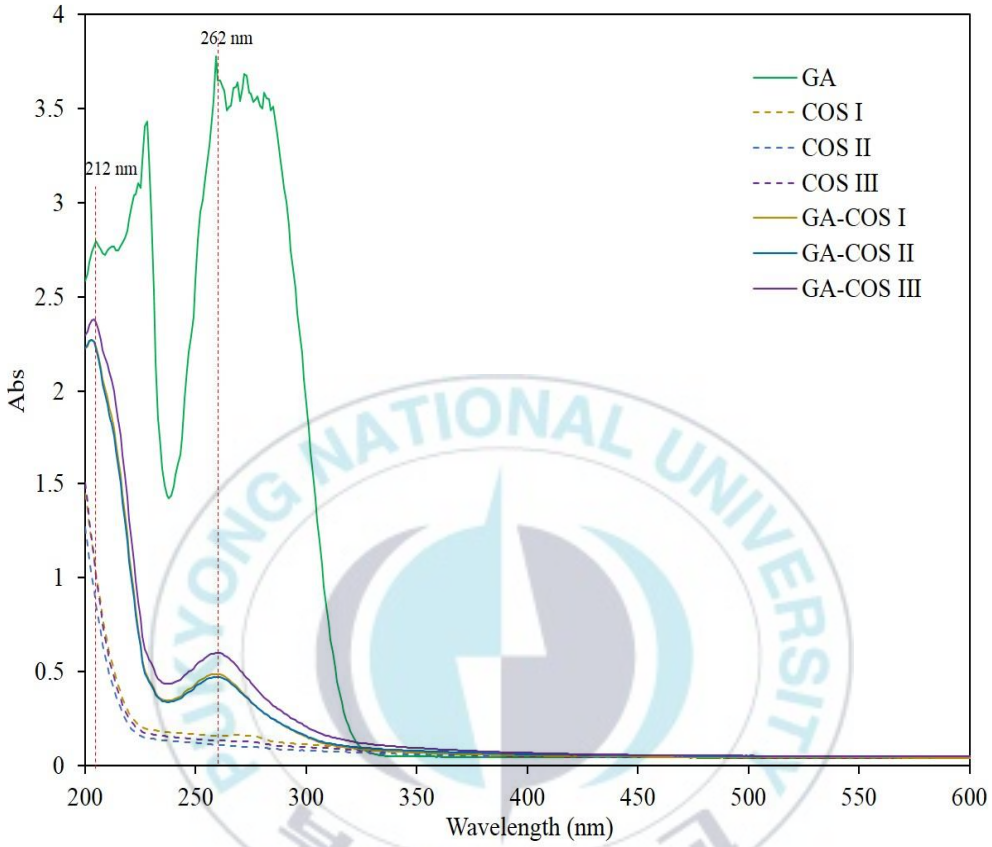


Fig. 7. The UV-visible absorption spectra of GA, COS I, II, III, GA-COS I, II and III.

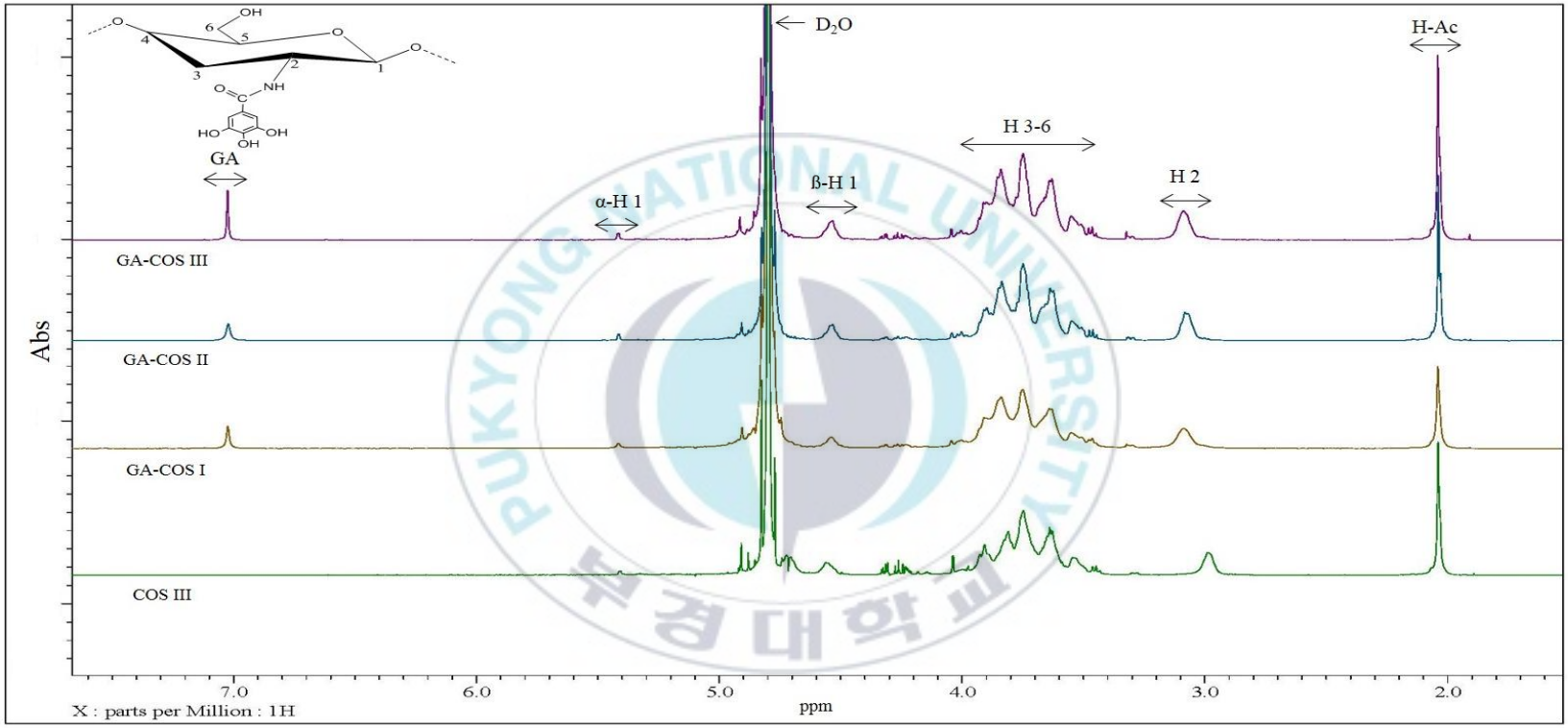


Fig. 8. ^{1}H-NMR (600 MHz, D_2O) spectra of COS III, GA-COS I, II and III

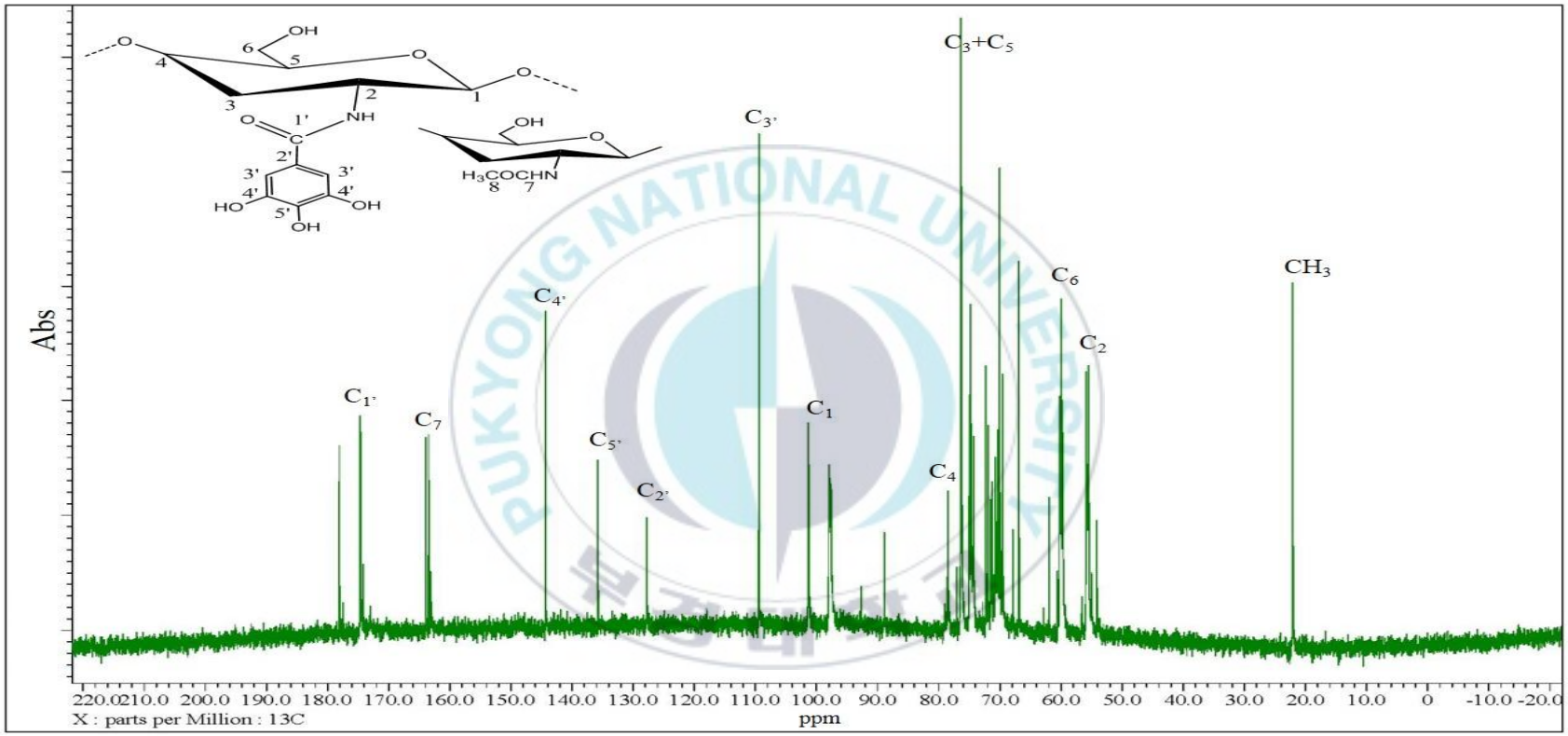


Fig. 9. ^{13}C-NMR (600 MHz, D_2O) spectrum of GA-COS III

Table 1. GA contents in GA-COS I, II and III.

Samples	mg GA/ g GA-COS*
GA-COS I	80.95 ± 0.53
GA-COS II	88.58 ± 8.12
GA-COS III	85.35 ± 7.54

3.2. Anti-oxidant activity of GA-COS

The IC_{50} values of samples on DPPH, ABTS radical and H_2O_2 are observed in Table 2. This experiment result indicated that anti-oxidant activity of all GA-COS was greatly improved through synthesis compared with all COS. Although IC_{50} values of GA-COS II were the lowest, GA-COS II did not indicate significant differences compared with GA-COS I and III.

3.3. Anti-bacterial activity of GA-COS

The MIC values indicated the quantitative anti-bacterial activity of all GA-COS and COS against *P.acnes* (Table 3). All GA-COS and COS showed to strongly inhibit the growth of *P.acnes* in that MIC values of all GA-COS and COS were less than 256 μg/mL. Moreover, GA-COS II and III showed slightly decreased MIC values than others.

The diameters of clear zones against *P.acnes* are also indicated in Table 3. The results denoted that synthesis of COS with GA improve anti-bacterial activity than just COS and GA-COS and COS with high molecular weight inhibit the growth of *P.acnes*. And, 1×10^{-5} N HCl (control) showed no anti-bacterial activity.

3.4. Physical properties of hydrogels

3.4.1. Water swelling property

The timed water absorption percentages of hydrogels are showed in Fig. 10. As a result, COS/PVA and GA-COS/PVA showed higher water swelling property compared

Table 2. 50% inhibition concentration (IC_{50}) values of COS I, II, III, GA-COS I, II and III on DPPH, ABTS radical and H_2O_2.

Samples	IC_{50} values (μg/ml)		
	DPPH radical*	ABTS radical*	Hydrogen peroxide*
COS I	> 500	> 500	> 500
COS II	> 500	> 500	> 500
COS III	> 500	> 500	> 500
GA-COS I	33.32±0.96	19.30±0.36	212.40±7.01
GA-COS II	32.49±0.70	18.95±0.61	207.03±5.60
GA-COS III	35.77±0.03	21.44±0.14	209.67±3.54

Table 3. Minimum inhibitory concentration (MIC) and clear zone of COS I, II, III, GA-COS I, II and III against *P.acnes*.

Strains	Control	COS I	COS II	COS III	GA-COS I	GA-COS II	GA-COS III
MIC (μg/mL)*	-[a]	256	128	128	128	64	64
Clear zone (mm)*	-[a]	13.73±1.15[b, c]	13.51±1.11[b]	15.05±0.72[b, c]	14.22±0.41[b, c, d]	15.58±0.18[b, c]	17.59±0.61[d]

to PPVA. In particular, COS/PVA and GA-COS/PVA rapidly absorbed much water than PPVA for the initial 1 h.

3.4.2. Gel fraction

The influence of COS and GA-COS on the gel fraction are observed in Fig. 11. The gel fraction data suggests relative crystallinity degree of hydrogels. The addition of COS and GA-COS resulted in decrease of gel fraction, showing following results. The gel fraction of PPVA was 81.50%, while the gel fractions of COS/PVA and GA-COS/PVA were 73.10 and 73.15%.

3.4.3. Hydrogel rheology

The relative mechanical property of hydrogels are observed in Fig. 12. This test evaluate the change in viscoelastic properties by changing the frequency. As a result, the G′ of hydrogels indicated almost no dependence with frequency, showing a good stability of hydrogels. Furthermore, PPVA showed the highest G′ at measured frequency.

3.5. Cell experiments

3.5.1. Cytotoxicity of hydrogels

The extratable and leachable study of hydrogels on NHDF, HaCaT and RAW 264.7 did not showed any evidence of cytotoxicity (Fig. 13). As the cell viability of control was 100%, the relative cell viability of PPVA, COS/PVA and GA-COS/PVA did not fall below 90%. These result denote that materials and fabricated products are biocompatible

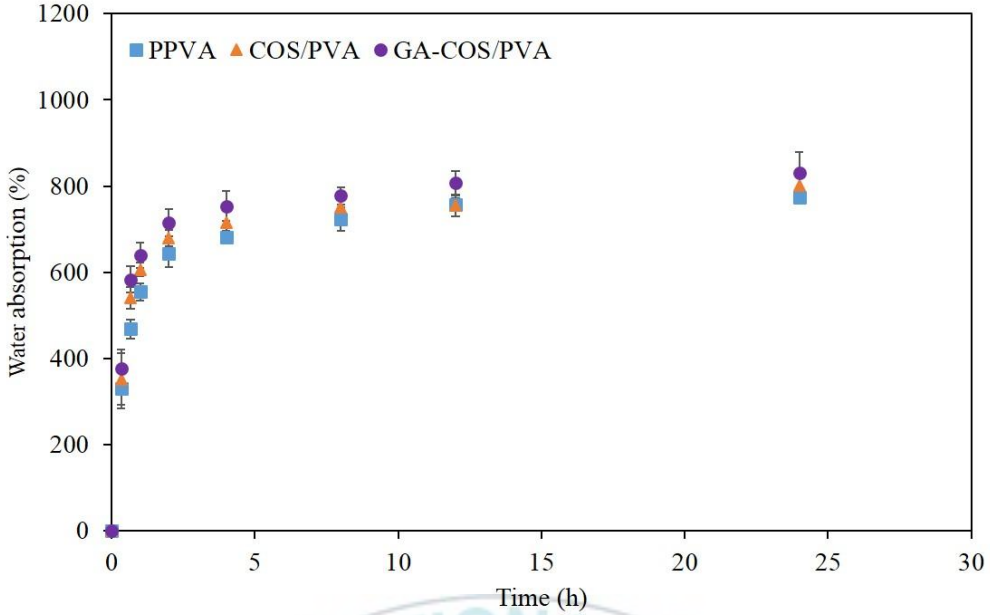


Fig. 10. The water swelling ration of PPVA, COS/PVA and GA-COS/PVA for 24 h at 37°C

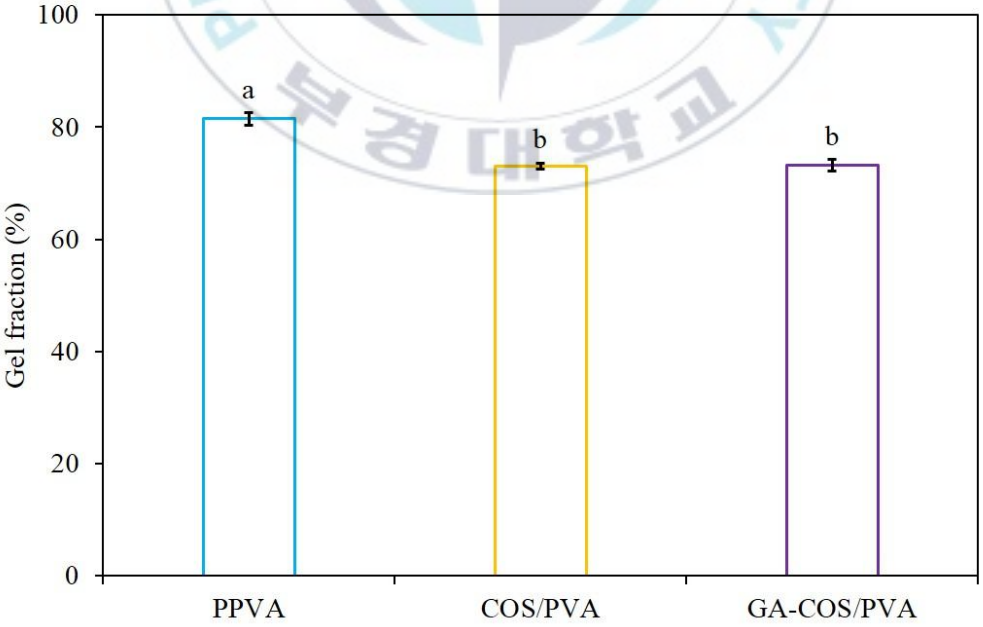


Fig. 11. The gel fraction on PPVA, COS/PVA and GA-COS/PVA

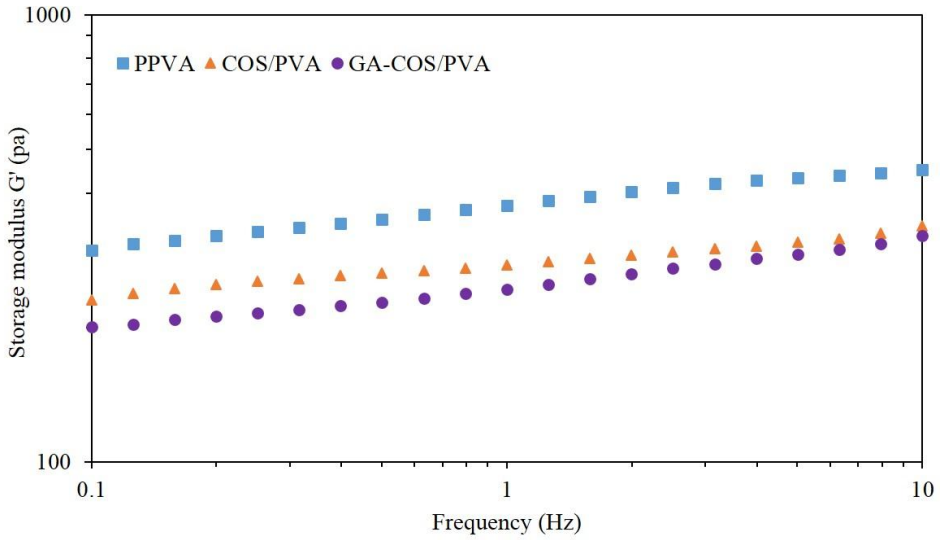


Fig. 12. The graph of the storage modulus vs frequency of PPVA, COS/PVA and GA-COS/PVA

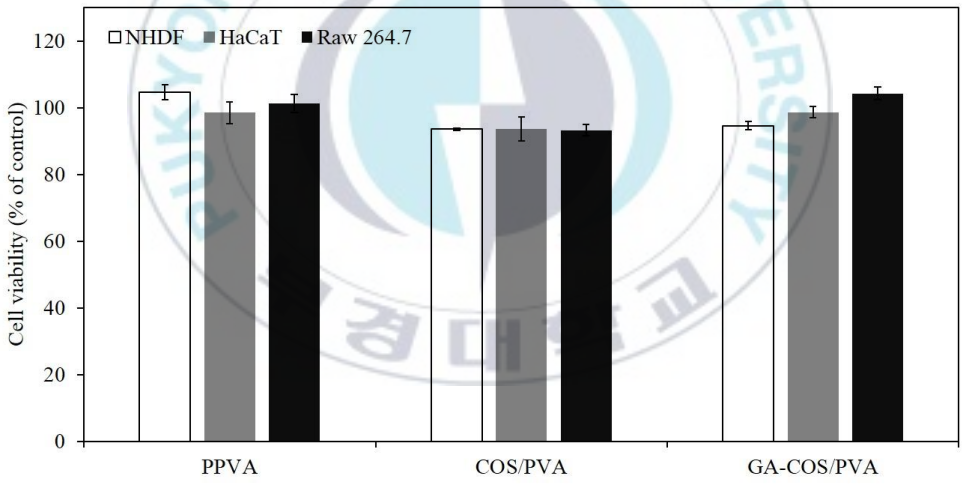


Fig. 13. The cytotoxicity of PPVA, COS/PVA and GA-COS/PVA evaluated by ISO 10993-5 standard with modifications.

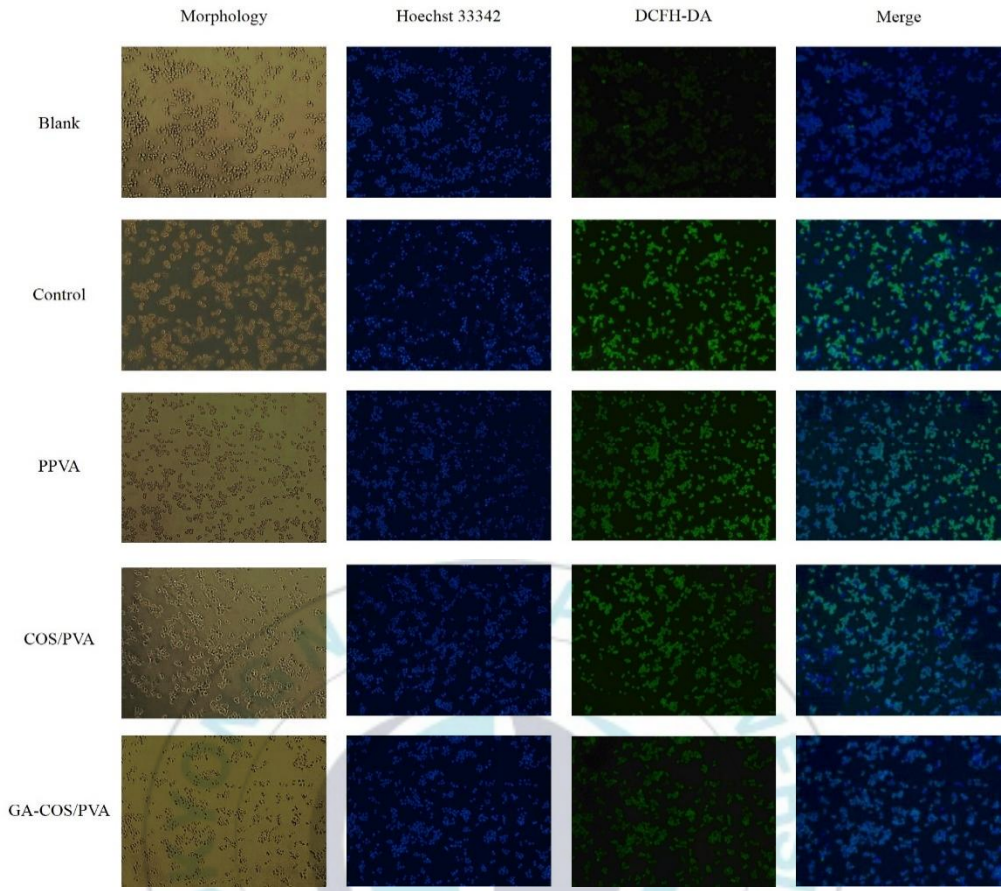
3.5.2. Inhibition effect on intracellular ROS generation

Images of ROS generation of RAW 264.7 incubated in the presence of H_2O_2 were monitored for further confirmation of intracellular ROS inhibition effect. As shown in Fig. 14, the GA-COS/PVA dramatically decreased fluorescence intensity of DCF when compared with control, PPVA and COS/PVA. In addition, cells stained with Hoechst 33342 showed that PPVA, COS/PVA and GA-COS/PVA were non-cytotoxic.

3.6. Anti-bacterial activity of hydrogels

The anti-bacterial activity of COS/PVA and GA-COS/PVA was evaluated by ASTM E2149-01. Fig 15 indicated the number of viable microorganisms present after incubation with hydrogels for fixed time. Control and PPVA did not show any activity against *P.acnes*. However, COS/PVA and GA-COS/PVA gradually decreased the number of survival microorganisms. In particular, GA-COS/PVA showed excellent anti-microbial property against *P.acnes*, although did not showed statistically significant differences.

(A)



(B)

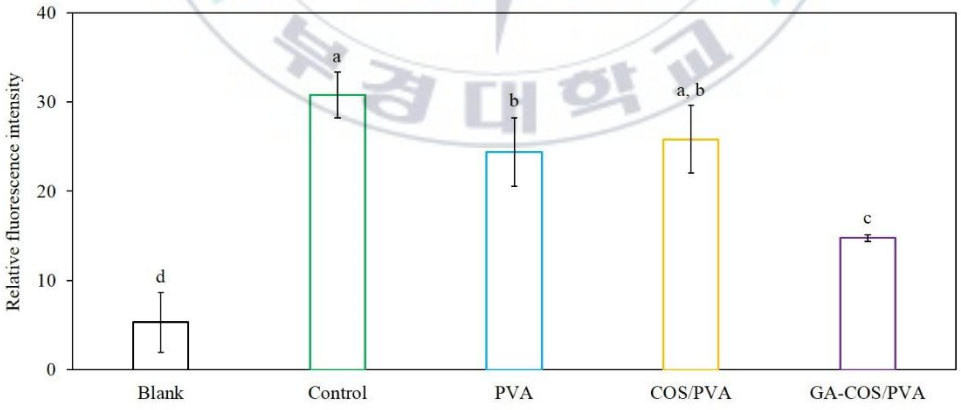
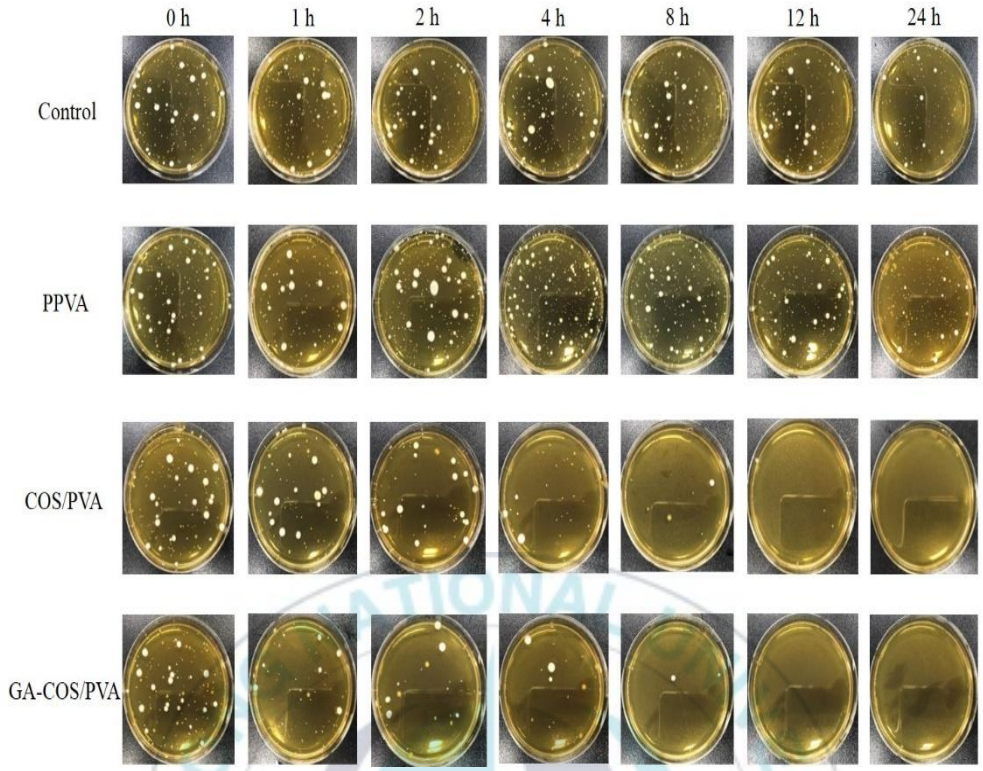


Fig. 14. The inhibitory effects of reactive oxygen species (ROS) generated in RAW 264.7. The images of RAW 264.7 stained with hoechst 33342 and DCFH-DA (A), the relative fluorescence intensity of DCF. Different letters on bars mean significant difference (p<0.05).

(A)



(B)

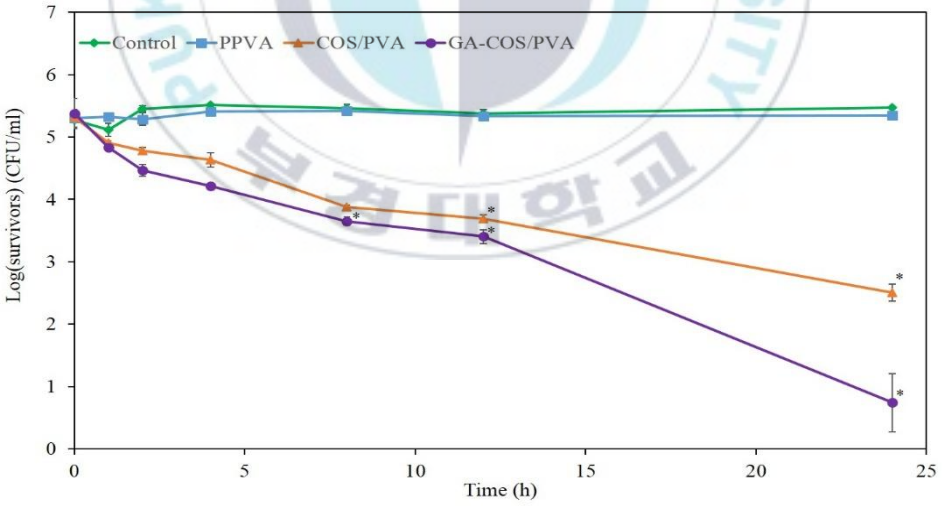


Fig. 15. The anti-bacterial activity of PPVA, COS/PVA and GA-COS/PVA. Images of survivor *P.acnes* (A), logarithmic plot of the survivor *P.acnes* number after incubation with PPVA, COS/PVA and GA-COS/PVA for fixed time (B). An asterisk means significant difference from control (p<0.05).

4. Discussion

COS and COS derivatives were already applied to biomedical products such as skin wound dressing, scaffolds for tissue regeneration and nanoparticle for cancer treatment, showing biocompatible and various bioactivities. There is rarely study reporting its application for acne vulgaris treatment, but nevertheless the demand of acne vulgaris care products with natural materials has been continually increased. In addition, previous studies reported that COS exhibit an anti-oxidant activity and used as health functional food (44,47). However, anti-oxidant activity of COS were observed to be remarkably weaker than that of antibiotics used for acne vulgaris treatment (48-50).

It is important factor to inhibit excessive ROS regenerated by neutrophils in acne vulgaris treatment in that superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), anti-oxidative enzyme, are decreased in patients with acne vulgaris and ROS continuously cause tissue injury at the inflammation site (10). Therefore, we synthesized COS with GA to improve anti-oxidant activity using the hydrogen peroxide mediated method. It has already been demonstrated that the chitosan conjugated with GA as same synthesis method and GA-COS synthesized by other method to improve anti-oxidant activity. However, these studies did not evaluate anti-bacterial activity against *P.acnes*. In addition, to our knowledge this is the first time GA-COS with molecular weight of less than 10 kDa are investigated as potential acne vulgaris treatment materials experimenting on cells and microorganism as compared with COS.

The synthesis of COS with GA successfully performed and the amount of conjugated GA on COS is no significant difference regardless of molecular weight (Table 1). It is very important that the anti-oxidant molecules on backbone polymer, because the anti-oxidant activity of conjugates depends on it. In present study, the values of GA-COS I, II and III (80.95, 88.58 and 85.35 mg GA/g GA-COS) are almost consistent with the

highest grafting degree value (88.53 mg GA/g GA-chitosan) of previous study suggested that the optimal mass ratio of chitosan to is 0.5:1, because the excess amount of free GA molecules inhibit the grafting process (34).

The MIC values and diameters of clear zones against *P.acnes* showed to be slightly affected by the addition of GA used as the anti-oxidant molecules while the IC_{50} values on scavenging DPPH, ABTS radicals and H_2O_2 were virtually based on GA (Table 2, 3). Interestingly, GA-COS showed more strong antibacterial activity against *P.acnes* As compared with MIC value of chitosan, chitosan-caffeic acid, chitosan-ferulic acid and chitosan sinapic acid previously reported (51). These results are due to complex anti-bacterial mechanisms considering hydrophilicity, charge density and adsorption capacity of the microorganism surface (52,53).

The physical characterization were examined to establish the water swelling and mechanical property of physical-crosslinking hydrogels. According to the previous reports, the mechanical property of hydrogel fabricated with only PVA gradually is improved during repeating the freezing-thawing cycle, because of abundant crystallization occurred between the hydroxyl groups of PVA chain in hydrogel (33,54). The mechanical property of PVA hydrogel is increased until six freezing-thawing cycle (55). But, PVA hydrogel fabricated through three freezing-thawing cycle is most suitable for use in biomedical application, because it display not only more flexibility and elasticity but also proper mechanical property (56-58). In addition, although COS/PVA and GA-COS/PVA showed to be less hard than PPVA, they showed the higher water swelling property which suggests the possibility to maintain a wet environment. It help to quickly heal wound occurred to skin surface pustules erupted.

Our study assure that the hydrogels do not affect fibroblast, keratinocyte and macrophage which present and play many role in skin and blood using indirect test (Fig. 13). Fibroblast produce extracellular matrix component and stimulate angiogenesis as

well as myofibroblast proliferation and activation during skin regeneration process (39). Keratinocyte is a typical cell constituting the epidermis. Its cell produces pro-inflammatory mediators to prevent invasion of pathogens (59). In particular, macrophage not only secrete pro-inflammatory mediators but also release H_2O_2 for phagocytosis (60,61). Therefore, in this study, RAW 264.7 was experimented to confirm inhibition effect on intracellular ROS generation. And, the results also suggest that GA-COS released from GA-COS/PVA significantly inhibited H_2O_2 excessively produced by macrophage in the anti-oxidative enzyme-poor environment (Fig. 14).

GA-COS and COS applied to fabrication of PVA hydrogel did not lose their anti-bacterial activity against *P.acnes*. It means that GA-COS/PVA and COS/PVA block to create an environment which continually sustains the colonization of *P.acnes* in skin. Furthermore, GA-COS/PVA and COS/PVA may reduce chronic inflammation by production of pro-inflammatory cytokines.

Even though further studies should focus on *in vivo* and clinical test, GA-COS/PVA overall showed promising physical-biological properties and a development possibility for acne vulgaris treatment.

5. Conclusion

In the current study, GA-COS with molecular weight of 1-3, 3-5, 5-10 kDa were synthesized via free radicals generated by ascorbic acid, which called as the hydrogen peroxide mediated method. And, GA-COS were experimented analysis of structure, anti-oxidant and anti-bacterial activity as compared with COS. As a result, GA-COS with weight of 5-10 kDa was chosen as a promising material for acne vulgaris treatment, indicating excellent anti-oxidant and anti-bacterial activity. Furthermore, PVA hydrogel which blended with candidate materials for acne vulgaris treatment applications was fabricated using the freezing-thawing method. GA-COS/PVA exhibited proper mechanical properties, cytocompatibility as well as anti-oxidant and anti-bacterial activity necessarily needed for acne vulgaris treatment. In conclusion, we suggest that GA-COS/PVA can be developed as the biomedical dressing and cosmetic product for patients suffer from acne vulgaris.

6. References

1. Raghupathi, W., and Raghupathi, V. (2014) Big data analytics in healthcare: promise and potential. *Health information science and systems* **2**, 3
2. Rademaker, M., Garioch, J., and Simpson, N. (1989) Acne in schoolchildren: no longer a concern for dermatologists. *Bmj* **298**, 1217-1219
3. Collier, C. N., Harper, J. C., Cantrell, W. C., Wang, W., Foster, K. W., and Elewski, B. E. (2008) The prevalence of acne in adults 20 years and older. *Journal of the American Academy of Dermatology* **58**, 56-59
4. Tan, J., and Bhate, K. (2015) A global perspective on the epidemiology of acne. *British Journal of Dermatology* **172**, 3-12
5. Fitz-Gibbon, S., Tomida, S., Chiu, B.-H., Nguyen, L., Du, C., Liu, M., Elashoff, D., Erfe, M. C., Loncaric, A., and Kim, J. (2013) Propionibacterium acnes strain populations in the human skin microbiome associated with acne. *Journal of investigative dermatology* **133**, 2152-2160
6. Mills, O. H., Criscito, M. C., Schlesinger, T. E., Verdicchio, R., and Szoke, E. (2016) Addressing Free Radical Oxidation in Acne Vulgaris. *The Journal of clinical and aesthetic dermatology* **9**, 25
7. Williams, H. C., Dellavalle, R. P., and Garner, S. (2012) Acne vulgaris. *The Lancet* **379**, 361-372
8. Rinnerthaler, M., Bischof, J., Streubel, M. K., Trost, A., and Richter, K. (2015) Oxidative stress in aging human skin. *Biomolecules* **5**, 545-589
9. Lynn, D. D., Umari, T., Dunnick, C. A., and Dellavalle, R. P. (2016) The epidemiology of acne vulgaris in late adolescence. *Adolescent health, medicine and therapeutics* **7**, 13
10. Basak, P. Y., Gultekin, F., and Kilinc, I. (2001) The role of the antioxidative

defense system in papulopustular acne. *The Journal of dermatology* **28**, 123-127

11. Bhate, K., and Williams, H. (2013) Epidemiology of acne vulgaris. *British Journal of Dermatology* **168**, 474-485
12. Das, R. K., Kasoju, N., and Bora, U. (2010) Encapsulation of curcumin in alginate-chitosan-pluronic composite nanoparticles for delivery to cancer cells. *Nanomedicine: Nanotechnology, Biology and Medicine* **6**, 153-160
13. Hayashi, K., and Ito, M. (2002) Antidiabetic action of low molecular weight chitosan in genetically obese diabetic KK-Ay mice. *Biological and pharmaceutical bulletin* **25**, 188-192
14. Kim, J.-H., Kim, Y.-S., Park, K., Kang, E., Lee, S., Nam, H. Y., Kim, K., Park, J. H., Chi, D. Y., and Park, R.-W. (2008) Self-assembled glycol chitosan nanoparticles for the sustained and prolonged delivery of antiangiogenic small peptide drugs in cancer therapy. *Biomaterials* **29**, 1920-1930
15. Zheng, L.-Y., and Zhu, J.-F. (2003) Study on antimicrobial activity of chitosan with different molecular weights. *Carbohydrate polymers* **54**, 527-530
16. Lee, D., Zhang, W., Shirley, S. A., Kong, X., Hellermann, G. R., Lockey, R. F., and Mohapatra, S. S. (2007) Thiolated chitosan/DNA nanocomplexes exhibit enhanced and sustained gene delivery. *Pharmaceutical research* **24**, 157-167
17. Lodhi, G., Kim, Y.-S., Hwang, J.-W., Kim, S.-K., Jeon, Y.-J., Je, J.-Y., Ahn, C.-B., Moon, S.-H., Jeon, B.-T., and Park, P.-J. (2014) Chitooligosaccharide and its derivatives: preparation and biological applications. *BioMed research international* **2014**
18. Fernandes, J. C., Tavaria, F. K., Fonseca, S. C., Ramos, Ó. S., Pintado, M. E., and Malcata, F. X. (2010) In vitro screening for anti-microbial activity of chitosans and chitooligosaccharides, aiming at potential uses in functional

textiles. *J Microbiol Biotechnol* **20**, 311-318

19. No, H. K., Park, N. Y., Lee, S. H., and Meyers, S. P. (2002) Antibacterial activity of chitosans and chitosan oligomers with different molecular weights. *International journal of food microbiology* **74**, 65-72

20. Eom, T.-K., Senevirathne, M., and Kim, S.-K. (2012) Synthesis of phenolic acid conjugated chitooligosaccharides and evaluation of their antioxidant activity. *Environmental toxicology and pharmacology* **34**, 519-527

21. Ngo, D.-H., Qian, Z.-J., Ngo, D.-N., Vo, T.-S., Wijesekara, I., and Kim, S.-K. (2011) Gallyl chitooligosaccharides inhibit intracellular free radical-mediated oxidation. *Food chemistry* **128**, 974-981

22. Vo, T.-S., Ngo, D.-H., and Kim, S.-K. (2012) Gallic acid-grafted chitooligosaccharides suppress antigen-induced allergic reactions in RBL-2H3 mast cells. *European Journal of Pharmaceutical Sciences* **47**, 527-533

23. Jones, V., Grey, J. E., and Harding, K. G. (2006) Wound dressings. *BMJ: British Medical Journal* **332**, 777

24. Queen, D., Orsted, H., Sanada, H., and Sussman, G. (2004) A dressing history. *International wound journal* **1**, 59-77

25. Boateng, J. S., Matthews, K. H., Stevens, H. N., and Eccleston, G. M. (2008) Wound healing dressings and drug delivery systems: a review. *Journal of pharmaceutical sciences* **97**, 2892-2923

26. Lee, T.-W., Kim, J.-C., and Hwang, S.-J. (2003) Hydrogel patches containing triclosan for acne treatment. *European journal of pharmaceutics and biopharmaceutics* **56**, 407-412

27. Hoffman, A. S. (2012) Hydrogels for biomedical applications. *Advanced drug delivery reviews* **64**, 18-23

28. Baker, M. I., Walsh, S. P., Schwartz, Z., and Boyan, B. D. (2012) A review of

polyvinyl alcohol and its uses in cartilage and orthopedic applications. *Journal of Biomedical Materials Research Part B: Applied Biomaterials* **100**, 1451-1457

29. Lee, K. Y., and Mooney, D. J. (2001) Hydrogels for tissue engineering. *Chemical reviews* **101**, 1869-1880

30. Kim, T. H., An, D. B., Oh, S. H., Kang, M. K., Song, H. H., and Lee, J. H. (2015) Creating stiffness gradient polyvinyl alcohol hydrogel using a simple gradual freezing–thawing method to investigate stem cell differentiation behaviors. *Biomaterials* **40**, 51-60

31. Huang, Y., Zhang, M., and Ruan, W. (2014) High-water-content graphene oxide/polyvinyl alcohol hydrogel with excellent mechanical properties. *Journal of Materials Chemistry A* **2**, 10508-10515

32. Tang, Y.-F., Du, Y.-M., Hu, X.-W., Shi, X.-W., and Kennedy, J. F. (2007) Rheological characterisation of a novel thermosensitive chitosan/poly (vinyl alcohol) blend hydrogel. *Carbohydrate Polymers* **67**, 491-499

33. Park, H.-H., Ko, S.-C., Oh, G.-W., Heo, S.-J., Kang, D.-H., Bae, S.-Y., and Jung, W.-K. (2017) Fabrication and characterization of phlorotannins/poly (vinyl alcohol) hydrogel for wound healing application. *Journal of Biomaterials Science, Polymer Edition*, 1-12

34. Hu, Q., Wang, T., Zhou, M., Xue, J., and Luo, Y. (2016) In vitro antioxidant-activity evaluation of gallic-acid-grafted chitosan conjugate synthesized by free-radical-induced grafting method. *Journal of agricultural and food chemistry* **64**, 5893-5900

35. Cho, Y.-S., Kim, S.-K., Ahn, C.-B., and Je, J.-Y. (2011) Preparation, characterization, and antioxidant properties of gallic acid-grafted-chitosans. *Carbohydrate Polymers* **83**, 1617-1622

36. Singleton, V. L., Orthofer, R., and Lamuela-Raventós, R. M. (1999) [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. *Methods in enzymology* **299**, 152-178

37. Nanjo, F., Goto, K., Seto, R., Suzuki, M., Sakai, M., and Hara, Y. (1996) Scavenging effects of tea catechins and their derivatives on 1, 1-diphenyl-2-picrylhydrazyl radical. *Free Radical Biology and Medicine* **21**, 895-902

38. Assaad, E., Maire, M., and Lerouge, S. (2015) Injectable thermosensitive chitosan hydrogels with controlled gelation kinetics and enhanced mechanical resistance. *Carbohydrate polymers* **130**, 87-96

39. Basu, A., Lindh, J., Ålander, E., Strømme, M., and Ferraz, N. (2017) On the use of ion-crosslinked nanocellulose hydrogels for wound healing solutions: Physicochemical properties and application-oriented biocompatibility studies. *Carbohydrate Polymers* **174**, 299-308

40. O'brien, J., Wilson, I., Orton, T., and Pognan, F. (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *The FEBS Journal* **267**, 5421-5426

41. Heo, S.-Y., Ko, S.-C., Kim, C. S., Oh, G.-W., Ryu, B., Qian, Z. J., Kim, G., Park, W. S., Choi, I.-W., and Phan, T. T. V. (2017) A heptameric peptide purified from Spirulina sp. gastrointestinal hydrolysate inhibits angiotensin I-converting enzyme-and angiotensin II-induced vascular dysfunction in human endothelial cells. *International Journal of Molecular Medicine* **39**, 1072-1082

42. Straccia, M. C., Romano, I., Oliva, A., Santagata, G., and Laurienzo, P. (2014) Crosslinker effects on functional properties of alginate/N-succinylchitosan based hydrogels. *Carbohydrate polymers* **108**, 321-330

43. Oh, G.-W., Ko, S.-C., Je, J.-Y., Kim, Y.-M., Oh, J., and Jung, W.-K. (2016) Fabrication, characterization and determination of biological activities of poly

(ε-caprolactone)/chitosan-caffeic acid composite fibrous mat for wound dressing application. *International journal of biological macromolecules* **93**, 1549-1558

44. Xie, M., Hu, B., Wang, Y., and Zeng, X. (2014) Grafting of gallic acid onto chitosan enhances antioxidant activities and alters rheological properties of the copolymer. *Journal of agricultural and food chemistry* **62**, 9128-9136

45. Ngo, D.-H., Qian, Z.-J., Vo, T.-S., Ryu, B., Ngo, D.-N., and Kim, S.-K. (2011) Antioxidant activity of gallate-chitooligosaccharides in mouse macrophage RAW264. 7 cells. *Carbohydrate polymers* **84**, 1282-1288

46. Lefay, C., Guillaneuf, Y., Moreira, G., Thevarajah, J. J., Castignolles, P., Ziarelli, F., Bloch, E., Major, M., Charles, L., and Gaborieau, M. (2013) Heterogeneous modification of chitosan via nitroxide-mediated polymerization. *Polymer Chemistry* **4**, 322-328

47. Xia, W., Liu, P., Zhang, J., and Chen, J. (2011) Biological activities of chitosan and chitooligosaccharides. *Food Hydrocolloids* **25**, 170-179

48. Miyachi, Y., Yoshioka, A., Imamura, S., and Niwa, Y. (1986) Effect of antibiotics on the generation of reactive oxygen species. *Journal of Investigative Dermatology* **86**, 449-453

49. Yen, M.-T., Yang, J.-H., and Mau, J.-L. (2008) Antioxidant properties of chitosan from crab shells. *Carbohydrate Polymers* **74**, 840-844

50. Sun, T., Zhou, D., Xie, J., and Mao, F. (2007) Preparation of chitosan oligomers and their antioxidant activity. *European Food Research and Technology* **225**, 451-456

51. Kim, J.-H., Yu, D., Eom, S.-H., Kim, S.-H., Oh, J., Jung, W. K., and Kim, Y.-M. (2017) Synergistic Antibacterial Effects of Chitosan-Caffeic Acid Conjugate against Antibiotic-Resistant Acne-Related Bacteria. *Marine Drugs*

15, 167

52. Chung, Y.-C., and Chen, C.-Y. (2008) Antibacterial characteristics and activity of acid-soluble chitosan. *Bioresource technology* **99**, 2806-2814

53. Chung, Y.-C., Wang, H.-L., Chen, Y.-M., and Li, S.-L. (2003) Effect of abiotic factors on the antibacterial activity of chitosan against waterborne pathogens. *Bioresource technology* **88**, 179-184

54. Berger, J., Reist, M., Mayer, J. M., Felt, O., and Gurny, R. (2004) Structure and interactions in chitosan hydrogels formed by complexation or aggregation for biomedical applications. *European Journal of Pharmaceutics and Biopharmaceutics* **57**, 35-52

55. Holloway, J. L., Lowman, A. M., and Palmese, G. R. (2010) Mechanical evaluation of poly (vinyl alcohol)-based fibrous composites as biomaterials for meniscal tissue replacement. *Acta biomaterialia* **6**, 4716-4724

56. Kim, J. O., Park, J. K., Kim, J. H., Jin, S. G., Yong, C. S., Li, D. X., Choi, J. Y., Woo, J. S., Yoo, B. K., and Lyoo, W. S. (2008) Development of polyvinyl alcohol–sodium alginate gel-matrix-based wound dressing system containing nitrofurazone. *International journal of pharmaceutics* **359**, 79-86

57. Kamoun, E. A., Kenawy, E.-R. S., Tamer, T. M., El-Meligy, M. A., and Eldin, M. S. M. (2015) Poly (vinyl alcohol)-alginate physically crosslinked hydrogel membranes for wound dressing applications: characterization and bio-evaluation. *Arabian Journal of Chemistry* **8**, 38-47

58. El Salmawi, K. M. (2007) Application of polyvinyl alcohol (PVA)/carboxymethyl cellulose (CMC) hydrogel produced by conventional crosslinking or by freezing and thawing. *Journal of Macromolecular Science, Part A: Pure and Applied Chemistry* **44**, 619-624

59. Graham, G. M., Farrar, M. D., Cruse-Sawyer, J. E., Holland, K. T., and Ingham,

E. (2004) Proinflammatory cytokine production by human keratinocytes stimulated with Propionibacterium acnes and P. acnes GroEL. *British Journal of Dermatology* **150**, 421-428

60. Nathan, C. F., and Root, R. (1977) Hydrogen peroxide release from mouse peritoneal macrophages: dependence on sequential activation and triggering. *Journal of Experimental Medicine* **146**, 1648-1662

61. van der Goes, A., Brouwer, J., Hoekstra, K., Roos, D., van den Berg, T. K., and Dijkstra, C. D. (1998) Reactive oxygen species are required for the phagocytosis of myelin by macrophages. *Journal of neuroimmunology* **92**, 67-75

7. Acknowledgement

호기롭게 시작한 연구실 생활을 뒤돌아보면 저에게 학문적인 지식을 쌓게 해줬을 뿐만 아니라 이 생활을 하지 않았으면 느끼지 못할 정말 많은 것들을 배우고 느낄 수 있었던 시간이라고 생각합니다.

먼저 지난 4년간 저에게 많은 관심과 격려를 하며 이끌어주신 정원교 교수님께 감사의 인사를 올립니다. 수많은 학회에 함께 참석하여 저의 견문을 넓히게 해주시고 뛰어난 연구자들과 같이 연구할 수 있게 지원해 주신 덕분에 저는 많은 것을 경험하고 배울 수 있었고 미래를 잘 설계할 수 있었습니다. 그리고 졸업논문 연구를 위해 실험도 하게 해주시고 미생물실험에 대해 많은 조언을 해주신 김영목 교수님, 하이드로겔에 대한 물리적 특성 연구에 대해 많은 조언 해주시고 항상 격려해주신 남승윤 교수님께도 감사의 인사 올립니다. 학부 시절부터 학생들을 위해 학업, 미래에 대해 상담해주시고 유익한 세미나와 행사들을 개최해주시며 언제나 의공학과를 위해 노력해주시는 오정환 교수님, 안예찬 교수님, 이명기 교수님, 강현욱 교수님, 박상혁 교수님께도 감사의 말씀 올립니다.

제가 하는 연구에 대해서 담임선생님처럼 일일이 봐주시며 잘 진행될 수 있게 많은 도움을 주시고 고민이 있을 땐 언제나 맛있는 것 사주시며 이야기 들어주신 고석천 박사님, 실장으로서 많은 후배를 이끌며 연구에 대한 아이디어가 넘치는 아이디어 뱅크 건우 행님, 후배들을 뒤에서 잘 챙겨주고 뭐든지 잘 해결해주는 만능 성영이 행님, 맨날 장난쳐도 잘 받아주고 같이 밥 먹으러 다니면 재미있는 민선이, 항상 의욕이 넘치며 많은 일을 해내는 태희, 언제나 묵묵히 자기 일을 하는 민성이, 지금도 잘하지만 앞으로 훨씬 더 잘할 근형이, 투덜대지만 맨날 이것저것 잘 도와주는 채영이, 빠르게 많은 것을 배워나가는 세창이, 맨날 밥 사주면서 편하게 대해준 영동이 행님, 연구실 생활에 대해 제일 먼저 친절하게 가르쳐준 수영이, 언제나 편하게 연락할 수 있는 태일이, 엉뚱하고 영어공부를 같이한 민규, 얄밉게 장난쳐도 마음은 참 착한 나루, 세상 속 편하게 살지만 의리 있고 세상 웃긴 창경이 행님, My best foreign friends Tinh, Chandika 모든 연구실원 분들에게 정말 큰 고마움을 표합니다. 그리고 생화학을 재미있게 잘 가르쳐주시고 항상 격려해주시는 박선주 교수님, 항상 유쾌하게 연구에 대해서 조언해주시는 강도형 박사님, 홍해삼에 대해 연구를 하게 지원해주시고 자신의 동생처럼 지켜봐 주시며 응원을 해주시는 허수진 박사님, 정말

자신의 후배처럼 챙겨주시고 우리도 할 수 있다는 것을 보여 주신 이승홍 교수님께도 감사의 말씀 올립니다. 동해심해생물에 대한 연구를 하게 도와주시고 연구선 승선 경험을 하게해주신 독도수산연구센터 김성태 연구관님, 윤상철 연구관님, 정경미 연구사님, 저에게 합성에 대해서, NMR 에대해서 정말 친절하게 가르쳐 주신 정희진 박사님, 언제나 만나면 반겨주며 실험실생활에 조언해주시는 류보미 박사님, 이정권 박사님, 김영상 박사님, 미생물에 관한 질문이나 실험을 부탁해도 항상 친절하게 도와주신 김송희, 김보금, 장유미 선생님께도 감사의 인사 올립니다.

욕심 많은 철부지 아들에게 언제나 헌신적인 사랑으로 남부럽지 않게 키워주신 부모님, 장난 심한 오빠랑 언제나 잘 놀아주는 하나밖에 없는 동생 혜란이에게 항상 사랑하고 감사하다는 말 전합니다. 1 년동안 응원과 칭찬으로 저를 최고로 만들어준 여자친구 윤혜에게도 사랑의 인사 전합니다.

저는 항상 많은 도움을 받으며 살아 온 것 같습니다. 항상 겸손한 자세로 받은 사랑과 관심 잊지 않고 베풀며 살겠습니다. 모든 분들께 감사의 인사 전해드립니다. 감사합니다.

박현호 올림.