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Gallic acid-grafted chitosan films as antioxidant food packaging

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To the Graduate Council:

I am submitting herewith a thesis written by Ping Guo entitled "Gallic acid-grafted chitosan films as antioxidant food packaging." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

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(Original signatures are on file with official student records.)

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ABSTRACT

Chitosan is the second most abundant natural polysaccharide in the nature. Due to its biodegradability and film forming ability, chitosan has the potential to be used as an alternative to petroleum-based polymers for food packaging. The presence of a primary amine as well as primary and secondary hydroxyl groups enable chitosan to be chemically modified with various functional groups. Gallic acid (GA) is a natural occurring antioxidant (AOX), which can be grafted to chitosan using by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). The first project deals with the effect of ethanol (EtOH) concentration on efficiency of grafting GA onto chitosan. Using pure deionized water as a sole solvent (0% EtOH), GA grafted to chitosan at the largest extent (285.9 mg GA/g chitosan). As the concentration of EtOH increased, the grafting efficiency proportionally decreased. The nuclear magnetic resonance (NMR) studies showed that the higher alcohol concentration inhibited successful grafting of GA by prohibiting the conversion of the intermediate O-acylisourea ester to the expected intermediate NHS-ester. To assist the NMR studies of solvent effect on grafting, the formation of GA-NHS ester was investigated in the second study by quantitative ^{1}H (proton) NMR reaction monitoring. Using a recently developed long-range heteronuclear single quantum multiple bond correlation (LR-HSQMBC), we were able to visualize a $^{5}J_{CH}$ (five-bond carbon-proton coupling) that confirmed the structure of the GA-NHS ester. The data showed that during grafting, a side reaction of crosslinking can occur as the hydroxyl groups of chitosan can be activated by EDC and coupled to the amino groups. In order to prevent the decreased solubility of chitosan caused by crosslinking, surface modification was introduced directly to chitosan films. The surface grafting was conducted utilizing EDC

and NHS and was confirmed by FTIR. Surface grafted GA-chitosan films exhibited excellent AOX activity, assessed as 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging efficiency and reducing power. When used as packaging material, the surface-grafted chitosan films had similar or better effect as polyethylene films protecting sunflower seeds from lipid oxidation. Overall, this research has developed surface-grafted GA-chitosan films with excellent antioxidant efficiency that may be utilized as multifunctional biodegradable food packaging material.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEME

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Chitosan

Chitosan, a co-polysaccharide of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose, has been considered as an alternative to synthetic polymers in food packaging due to its biodegradability and film forming ability.[1-3] Chitosan is produced by the deacetylation of chitin. Chitin is commercially isolated from a variety of sources, predominantly marine, such as the shells of several crustacean species, but it is also present in the exoskeleton of insects and cell wall of fungi. Chitin is further processed by alkaline hydrolysis under harsh conditions in order to remove the acetyl groups.[4] When the degree of deacetylation of chitin reaches about 50%, it becomes soluble in aqueous acidic media and is called chitosan.[5] The interest in chitosan is constantly increasing due to its biocompatibility,[6] biodegradability,[7] antibacterial properties,[8] and affinity for many proteins.[9] Chitosan has been commercially used in water purification,[10-12] evaluated as an antimicrobial food packaging[2, 13, 14] and as a carrier in drug delivery systems.[15-17] The presence of amino groups enables chitosan to adsorb metal cations by chelation, and electrostatically attract metal or dye anions to the protonated amino groups in acidic solution[18, 19], as well as to be chemically modified.[20, 21]

One of the major advantages of chitosan is that it is soluble in dilute aqueous acid, and such solutions can be cast into films and fibers due to the linear structure of chitosan molecules.[4, 5] Chitosan films have a low gas permeability,[22] good mechanical properties,[23] excellent metal binding potential,[20] and with chitosan's intrinsic antimicrobial efficiency,[24] may serve as multifunctional active packaging. Therefore, chitosan was chosen to be modified to produce biodegradable antioxidant packaging in

this research.

Carbodiimide Grafting of Chitosan

There is an increased interest in chemically modifying chitosan by grafting it with phenolic acids in order to introduce primary antioxidant properties and thus extend the shelf-life of packaged food.[1, 3, 25] Modification of chitosan has been achieved by simple mixing or coating,[26, 27] or by physiochemical and biochemical methods such as irradiation,[28, 29] and enzymatic[30-32] or free radical reactions.[1, 25, 33, 34] Mixing is an easy and fast way but with a high possibility for antioxidants to be lost from the films by volatilization or leaching.[25] Radiation introduces covalent bonding between the AOXs and chitosan but often causes degradation of either the polymer or phenolic acid, or both. Radiation induces polymer degradation via chain scission, resulting in cracking of the surface and loss of mechanical properties,[25, 35] while phenolic acid may be degraded by hydroxyl radicals generated during radiolysis of water.[36] Enzymatic methods using laccase,[31] tyrosinase,[37] and horseradish peroxidase[38] have been used to functionalize chitosan with phenolic compounds, but can catalyze oxidation of phenolics,[39] and thus reduce their AOX properties. In contrast, carbodiimide, extensively used in amidation of proteins[40] offers an alternative chemical approach for the modification of chitosan.[1, 33]

Carbodiimides are widely used in peptide synthesis, [41, 42] and in nano(bio)technology for the immobilization of biomolecules on surfaces and nanoparticles as efficient coupling reagents.[43-46] They are able to mediate the formation of strong amide bonds

between amino and carboxyl groups under mild conditions. Additionally, studies have shown that N-ethyl-N-(dimethylaminopropyl)-carbodiimide (EDC) can mediate ester bonds between carboxyl and hydroxyl groups.[1, 33, 47] Notably, EDC is among the most popular carbodiimide reagents because it is reasonably inexpensive, non-toxic, highly soluble in aqueous solution and the corresponding product, urea, can be easily removed after the reaction to produce clean products.[48-50] EDC reacts with carboxylic acid to form an O-acylisourea, which has an extremely short half-life and rapidly undergoes hydrolysis or rearranges to a more stable N-acylisourea.[50] Adding N-hydroxysuccinimide (NHS) under facile conditions, allows NHS to react with O-acylisourea to form relatively stable intermediates.[50] These intermediates are hydrophilic active esters, aminoacyl esters, and since their hydrolysis rate is much slower than the rate of their reaction with primary amino groups,[51] addition of NHS increases the yield of amidation.

Given the widespread of this method, EDC/NHS could be thought to be a well-established protocol leading an efficient amidation. However, an important number of parameters for this method vary greatly between studies.[45, 52, 53] For instance, the ratio between EDC and NHS span in a wide range from 20/1 to 1/20;[52] the pH for this reaction has been reported in the range 4.5-7.2;[52, 54] and the temperature includes 25 °C and 0 °C.[33, 52, 54] In all of these studies and in many others investigating amidation using ECD/NHS, aqueous ethanol often served as a solvent throughout the reaction. However, the concentration of EtOH was not consistent across these reports, and it is not clear how the EDC/NHS coupling reaction is affected by EtOH concentration, or how the EtOH concentration affects the grafting efficiency.[33, 55, 56] Furthermore, Nam et al.[57] used EDC/NHS for collagen cross-linking and reported that the crosslinking

rate increased as the EtOH concentration in the solvent increased up to 0.12 M (6.9% v/v) but decreased as the concentration was increased further. Therefore, we investigated the effect of EtOH concentration (0 - 75% v/v) on efficiency of grafting GA to chitosan utilizing EDC and NHS.

Antioxidant Active Packaging

Lipid plays an important role in food quality in terms of nutrition, mouth feel, satiety, and health promotion.[58] However, lipid oxidation is often a major problem in food processing and storage due to off-odors, off-flavors, texture and/or color changes, and nutrition losses, leading to a significant reduction in product shelf life and ultimately product loss.[58-60] To protect food from lipid oxidation, antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and ethylenediamine tetraacetic acid (EDTA), are commonly used in controlling oxidative reaction in food systems. However, these synthetic antioxidants have increasingly been negatively perceived, while natural food products are becoming more desirable by consumers. Even though natural antioxidants like vitamin C and tocopherol are more acceptable for consumers, a large amount of antioxidant is needed to impart the same effect as synthetic antioxidants. Both synthetic and natural antioxidant food preservatives may affect the food quality. For instance, when ferulic acid was used as a food preservative, thermal decarboxylation of ferulic acid formed 4-vinylguaiacol during cooking, the main contributor of off-flavors in many cooked products.[58, 61] Therefore, active packaging with antioxidant efficiency is becoming more of interest as an alternative way for efficient food preservation.[62] By incorporating antioxidants into packaging

material, antioxidant active packaging goes beyond the traditional role of packaging, not only providing an inert barrier to external environment, but also executing antioxidant activities.[58]

Recent studies on antioxidant active packaging have reported different ways to incorporate antioxidants into packaging systems, including independent sachet packages,[62] physical coating on packaging material surface,[63] mixing into main packaging polymer matrix,[2, 64] and covalent immobilization[1] onto packaging material. However, sachets are not applicable for liquid or high humidity food products since the direct contact between the liquid and the sachets may cause the spillage of sachet contents.[65] Mixing antioxidants into packaging polymer matrix is an easy and fast way but the migration of the antioxidant from the packaging to food products may affect food quality.[25] Covalent immobilization of antioxidant onto packaging material provides the most stable linkage between the material and antioxidants. Such bound antioxidants have a low possibility to migrate from the package to the food.[58]

Gallic acid (GA, 3,4,5-trihydroxy benzoic acid) is a naturally occurring antioxidant. GA and its derivatives form a large family of plant secondary polyphenolic metabolites, and are normally present in fruits, vegetables, nuts, tea, etc.[66-68] GA derivatives, such as propyl gallate[69] and eppigallocatechin gallate,[70] have been widely used as food additives to prevent oil rancidity. Their antioxidant activity is achieved by direct termination of free radicals by rapid donation of hydrogen atoms or electrons, so they are classified as primary AOXs.[71] With three phenolic hydroxyl groups in its structure, GA exhibits strong AOX activity, while the carboxyl group enables its grafting to various matrices, including collagen and chitosan, through

amidation and/or esterification reactions.[40, 47] Using EDC/NHS, GA can be activated to form GA-NHS ester, which then reacts with the amino and hydroxyl groups on chitosan to produce GA-grafted chitosan.[1, 33, 47] Therefore, in this study, GA was grafted onto chitosan to produce antioxidant packaging via EDC/NHS.

NMR Investigation of EDC/NHS Activation of GA

Given the fact that many studies were focused on improving the efficiency of amidation via EDC/NHS, the overall efficiency of incorporation of functional groups could not be improved beyond 25% by varying the level of activation.[50, 52] Furthermore recent studies[45, 53, 72] have highlighted the complexity of this reaction by showing different possible paths to form the NHS-ester. However, the spectroscopic evidence of the NHS ester of benzoic acids is often omitted. To gain insight into the cause of the low yield, quantitative ^{1}H NMR, reaction monitoring and structure elucidation by NMR allowed us to investigate the interfering reaction during the activation of GA and the formation of GA-NHS ester.

NMR is a spectroscopic technique based on the magnetic properties of atomic nuclei. It was discovered by American physicists Bloch[73] and Purcell[74] in 1945. As NMR spectroscopy provides useful information about structural, conformational and dynamic analysis of molecules in solution molecular structure, kinetic analysis of reactions and diffusion processes, it has rapidly become the most powerful non-destructive analytical tool in chemistry.[75] In biochemistry, NMR spectroscopy has been applied to structure elucidation of proteins,[76] nucleic acid[77] and viruses.[77] For biomedical research,

NMR can be used to living tissues and organisms which allows the study of their physiology and metabolism *in vivo*.[78] The application of NMR in food science was delayed until the 1980s, primarily because of the complexity of food systems, lack of scientific expertise, high cost of equipment, and the absence of NMR instruments or methods designed specifically for food analysis.[79] With the development of NMR instrumentation and programs to collect and analyze the data, NMR's applicability has been growing rapidly in food science and technology. NMR food-related research has covered various fields including food compositional (e.g. water, fat and protein) analysis,[80-83] identification and structure determination of food compounds, such as fructooligosaccharides from roots and leaves of *Stevia rebaudiana* (Bert.) Bertoni,[84] inspection of microbiological, physical and chemical quality,[85-87] food authentification,[88, 89] and on-line monitoring of food processing.[90]

Another important situation for application of NMR in food science is comparable to that in chemistry, as a tool for analytical determinations.[91-93] Interest in quantitative ^{1}H NMR continues to increase because it offers the selectivity without separation, accuracy based on the technical progress of NMR programs, and is not limited by the polarity of compounds.[94, 95] The mechanism of quantitative ^{1}H NMR is based on that the integral of corresponding peak on the spectrum is proportional to the number of nuclei that generates the signal.[95] The absolute concentration of analyte can be calculated using the intensity ratio between the analyte and standard compound with known concentration.[94]

Reaction monitoring by NMR is a powerful tool for mechanistic elucidation of chemical reactions.[96-100] This analytical technique provides information not only for the concentration of each component in the reaction mixture, but also confirms the

presence of various intermediates and side products forming and disappearing during the reaction.[96, 101] These advantages make reaction monitoring by NMR an excellent method to gain insight into the reactions found in engineering, chemical, and biocatalyzed processes.[96, 97, 99, 101] For EDC/NHS reaction, reaction monitoring by NMR allows us to confirm the formation of all the products from this activation process. However, to investigate the structure and identify the expected NHS-ester, a long-range correlation experiment was necessary.[102]

Given the expected structure of the NHS-ester (e.g. GA-NHS ester in Figure 1.1), it becomes clear that to prove the structure of this molecule, it is necessary to visualize a $^{5}J_{CH}$ from H11/12 to C7 using a recently developed NMR experiment, long-range heteronuclear single quantum multiple bond correlation (LR-HSQMBC).[102] LR-HSQMBC is based on the heteronuclear single quantum multiple bond correlation (HSQMBC) pulse sequence,[103] started with the G-BIRD$_{R,X}$-HSQMBC pulse first published in 2002,(*104*) and added both a refocusing step, and decoupling during acquisition.[102] These additions as well as the conversion of antiphase $2I_yS_z$ component to the nonobservable $2I_yS_y$ through a 90° pulse on the X nucleus (e.g. Carbon) prior to acquisition, further increased the sensitivity of the experiment to acquire a $^{5}J_{CH}$ even $^{6}J_{CH}$.[102]

By using quantitative 1H NMR, reaction monitoring and structure elucidation by NMR, LR-HSQMBC in particular, we were able to investigate and confirm the formation NHS-ester, as well as the interfere reaction caused by solvent and further to improve the solvent condition for grafting GA onto chitosan via EDC/NHS.

Surface Grafting of Chitosan Films

Several surface modification techniques have been developed to do the surface functionalization, mainly including ionized gas treatments, UV irradiation, and wet chemistry.[58] The most commonly used ionized gas treatment is plasma.[58] Plasma treatment increases the amount of active groups on the films, resulting in the increased surface energy and the reactive film surface.[105] The surface modification produced by UV irradiation has been applied to several polymers.[106, 107] UV light can create functional groups or free radicals on the polymer surface to further initiate graft polymerization of functional monomers.[58] In wet chemical methods, polymers are treated with liquid reagents to graft functional groups onto the surface of packaging material.

When EDC is used to graft GA onto chitosan, sufficient EDC is added to improve the efficiency of grafting. However, as a side reaction, the hydroxyl groups of chitosan can be activated by EDC and coupled to the amino groups, easily leading to crosslinking of the polymer[108] and reducing the solubility of the GA-grafted chitosan in aqueous acetic acid. This, in turn, makes it difficult to form a homogeneous film forming solution and produce chitosan films. In this context, surface modification of pre-produced pure chitosan films in GA and EDC/NHS reaction mixture can be an alternative to produce GA-grafted chitosan films as antioxidant active packaging.

Overall Goals and Objectives

The overall goal of the research was to develop antioxidant active packaging: gallic acid-grafted chitosan films. The first objective was to improve the solvent condition for grafting gallic acid onto chitosan using EDC/NHS. This was achieved by comparison of grafting efficiency in aqueous ethanol solutions with different concentrations, and NMR investigation of EDC/NHS activation of gallic acid in methanol-d_4/D_2O solution at various concentrations. The second objective was to investigate the formation of gallic acid-NHS ester in the process of EDC/NHS activation of gallic acid. This was achieved using ^1H NMR reaction monitoring and structure elucidation by LR-HSQMBC. The third objective was to develop a method for surface grafting of chitosan films via EDC/NHS and evaluate these films for physical, antioxidant properties and the ability to protect food from lipid oxidation. Storage studies were carried out to evaluate the grafted films as packaging using sunflower seeds. Lipid oxidation of the sunflower seeds was determined as peroxide value, conjugated dienes and trienes, and TBARS.

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Appendix: Chapter I

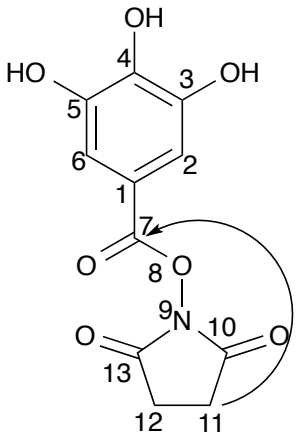

Figure 1.1 Structure of gallic acid-NHS ester

CHAPTER II

THE EFFECT OF SOLVENT COMPOSITION ON GRAFTING GALLIC ACID ONTO CHITOSAN VIA CARBODIIMIDE

This chapter is a lightly revised version of a paper by the same title submitted to Green Chemistry by Ping Guo,[a] John D. Anderson,[b] Joseph J. Bozell [b] and Svetlana Zivanovic[a*]

Ping Guo was responsible for experiment design, setting up experiments, discussing with advisor Dr. Zivanovic, and writing manuscripts.

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Abstract

Chitosan, a natural polymer with potential use in food packaging, exhibits significant antimicrobial efficiency due to its positive charge and considerable antioxidant (AOX) activity due to ability to bind metal ions (secondary AOX). The primary AOX activity can be introduced by grafting of phenolic compounds to its amino and/or hydroxyl groups. Phenolic acids are efficient AOXs, acting by rapid donation of a hydrogen atom or electron terminating free radicals and resulting in relatively stable phenoxy radicals. The objective of this study was to investigate the effect of ethanol (EtOH) concentration (0%, 25%, 50%, and 75% in water) on efficiency of grafting gallic acid (GA) onto chitosan in the presence of 1-ethyl-3-(3-dimethylaminopropyl) - carbodiimide (EDC)/N-hydroxysuccinimide (NHS). The grafting was confirmed by FTIR and the efficiency was quantified as Folin's total phenolics. The AOX properties of grafted chitosans were assessed as DPPH scavenging activity and as reducing power using K-ferricyanide. When pure deionized water was used as a sole solvent (0% EtOH), GA grafted to chitosan at the largest extent (285.9 mg GA/g chitosan) and the grafted chitosan had the highest DPPH activity and reducing power. As the concentration of EtOH increased, the grafting efficiency, DPPH activity, and reducing power of the grafted GA-chitosan proportionally decreased. The nuclear magnetic resonance (NMR) studies showed that the higher alcohol concentration inhibited successful grafting of GA by prohibiting the conversion of the intermediate O-acylisourea ester to a more stable but reactive NHS ester. The results confirm that the concentration of EtOH in grafting solution significantly affect grafting efficiency of GA on chitosan.

Introduction

Food packaging with antioxidant (AOX) properties can extend food shelf-life and improve safety. Developments in this field are continuously evolving in response to the growing demand for multifunctional active packaging.[109-112] Chitosan, a co-polysaccharide of 2-acetamido-2-deoxy-β-D-glucose and 2-amino-2-deoxy-β-D-glucose, has been considered as an alternative to synthetic polymers in food packaging due to its biodegradability and film forming ability.[1-3] Chitosan is produced by the deacetylation of chitin, obtained from crustacean shells left as a waste in the seafood industry. The presence of amino groups enables chitosan to chelate metal ions[18, 19] and to be chemically modified.[20, 21] Chitosan has been commercially used in water purification,[10-12] and evaluated as an antimicrobial food packaging[2, 13, 14] and as a carrier in drug delivery systems.[15-17] Chitosan films have a low gas permeability,[22] good mechanical properties,[23] excellent metal binding potential,[20] and with chitosan's intrinsic antimicrobial efficiency,[24] may serve as multifunctional active packaging. Furthermore, there is an increased interest in chemically modifying chitosan by grafting it with a phenolic acid in order to introduce primary antioxidant properties and thus extend the shelf-life of packaged food.[1, 3, 25]

Modification of chitosan films has been simply achieved by mixing or coating,[26, 27] or by physiochemical and biochemical methods such as irradiation,[28, 29] or enzymatic[30-32] and free radical reactions.[1, 25, 33, 34] Mixing is an easy and fast method but with a high possibility for antioxidants to be lost from the films by volatilization or leaching.[25] Radiation introduces covalent bonds between the AOXs and chitosan but often causes degradation of either the polymer or phenolic acid, or both. Radiation

induces polymer degradation via chain scission, resulting in cracking of the surface and loss of mechanical properties,[25, 35] while phenolic acid may be degraded by hydroxy radicals generated during radiolysis of water.[36] Enzymatic methods using laccase,[31] tyrosinase,[37] and horseradish peroxidase[38] have been applied to functionalize chitosan with phenolic compounds, but can catalyze oxidation of phenolics,[39] and thus reduce their AOX properties. In contrast, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), extensively used in amidation of proteins[40] offer an alternative chemical approach for the modification of chitosan.[1, 33] This method requires only mild reaction conditions, does not exhibit the disadvantages of the other procedures, and uses reagents (EDS and NHS) that can be easily removed after the reaction to produce clean products.

Gallic acid (GA, 3,4,5-trihydroxy benzoic acid) is a naturally occurring antioxidant. GA and its derivatives form a large family of plant secondary polyphenolic metabolites, and are normally present in fruits, vegetables, nuts, tea, etc.[66-68] GA derivatives, such as propyl gallate[69] and eppigallocatechin gallate,[70] have been widely used as food additives to prevent oil rancidity. Their antioxidant activity is achieved by direct termination of free radicals by rapid donation of hydrogen atoms or electrons, so they are classified as primary AOXs.[71] With three phenolic hydroxyl groups in its structure, GA exhibits strong AOX activity, while the carboxyl group enables its grafting to various matrices, including collagen and chitosan, through amidation and/or esterification reactions.[40, 47]

GA can be grafted to chitosan via amide or ester linkages by first activating the carboxyl group of GA through conversion to amino or hydroxyl-reactive intermediate

via EDC/NHS.[1, 33] As indicated in Scheme 2.1, GA **1** reacts with EDC **2** to form the hydrolytically unstable O-acylisourea **3** (t½ = seconds in aqueous solution).[52, 113] However, in the presence of NHS **4**, the longer lived GA-NHS ester **5** (t½ = hours in aqueous solution) is formed[114] and serves as an activated ester that reacts further with $–NH_2$ and/or –OH on chitosan **7** to form GA-grafted chitosan **8**.[33]

Although the reaction is simple, it is time consuming and results in relatively low grafting efficiency. In order to improve the reaction, studies have been conducted that alter the EDC/NHS ratio,[45, 72, 115] the concentration of all components,[1] or the pH of the reaction.[52] Nonetheless, the reaction still suffers from a low grafting efficiency. In all of these studies and in many others investigating grafting of GA or other carboxylic acids to chitosan using ECD/NHS, aqueous ethanol served as a solvent throughout the reaction. However, the concentration of EtOH was not consistent across these reports, and it is not clear how is the EDC/NHS coupling reaction is affected by EtOH concentration, or how the EtOH concentration affects the grafting efficiency.[33, 55, 56] Furthermore, Nam et al.[57] used EDC/NHS for collagen cross-linking and reported that the crosslinking rate increased as the EtOH concentration in the solvent increased up to 0.12 M (6.9% v/v) but decreased as the concentration was increased further. Here we present the effect of EtOH concentration (0 - 75% v/v) on efficiency of grafting GA on chitosan utilizing EDC and NHS, and chemistry underlying the change in the reaction.

Experimental

Materials and equipment

Chitosan with an average molecular weight of 307 kDa and 80% degree of

deacetylation (DDA), was donated by Primex. EDC (99.8% purity) and NHS (98% purity) were purchased from Acros Organics. GA was purchased from Sigma-Aldrich.

Purification of chitosan

Chitosan flakes were dissolved in 1w/w% acetic acid to form a 1w/w% chitosan solution. The solution was stirred overnight, filtered through Miracloth®, and chitosan was precipitated by adjusting the pH to ~10. The precipitate was washed with deionized water until the washing solution is neutral, followed by freeze-drying. Purified chitosan was kept in a desiccator at room temperature until needed.

Synthesis of GA-grafted chitosan

GA-grafted chitosan was prepared using a modified literature method.[33] GA (0.500 g, 3 mmol), EDC (0.580 g, 3 mmol) and NHS (0.340 g, 3 mmol) were mixed as solids, added to 20 mL of various concentrations of aq EtOH, and stirred in an ice bath for 1 h. Chitosan (0.32 g, 1.18 μmol), dispersed in 30 mL aq. EtOH of the same concentration, was added to the solution, and additionally stirred for 0.5 h in ice-bath followed by 6 h stirring at room temperature (standard procedure). After the grafting was completed, the product was centrifuged at 3,315 g for 20 min, washed 3 times with 50 mL aliquots of 75% EtOH, and freeze dried. To test the effect of EtOH concentration on grafting efficiency, four concentrations (0, 25, 50, and 75% v/v) of aq. EtOH were used. To test the effect of time on grafting efficiency, 25% EtOH was used as solvent and the last step (stirring in the presence of chitosan) was varied between 2, 6, 12 and 24 h. The concentration of residual GA was determined by washing grafted chitosan (25% EtOH, 6 h) 7 times with 50 mL 75% aq. EtOH and analyzing the wash for total phenolics.

Confirmation of grafting and characterization of GA-grafted chitosan

Chitosan, grafted chitosan, and a mixture of GA and chitosan (mix) prepared in the same ratio as found in grafted chitosan were ground to a fine powder with a mortar and pestle, mixed with KBr, and pelleted. FTIR spectra were acquired on the pellet (Nicolet NEXUS 670, Thermo, Madison, WI) between 500 and 4000 cm^{-1}, with 128 scans and resolution of 4 cm^{-1}.

Solubility of grafted chitosan in 1% acetic acid. Solubility of 0.1% w/w pure chitosan, grafted chitosan, and mix in 1% acetic acid was assessed as transmittance (T%) at 600 nm using a spectrometer (UV-2101PC Shimadzu, Columbia, MD)[1], with transmittance of 100% indicating complete solubility.

Determination of total phenolics content. Total phenolics content was determined by Folin-Ciocalteau method [116] with modification[1]. Briefly, the grafted chitosans were solubilized by sonication (Bradson 1510, Brason Ultrasonics Corp., Danbury, CT) in 0.25% acetic acid to give a 0.025% solution of dissolved chitosan. 1 mL of this solution was added to 7 mL DI water with 1 mL Folin-Ciocalteau reagent. After 3 min, 12.4% sodium carbonate solution was added to the mixture, and the solution was vortexed. The mixture was kept at 40°C for 30 min, after which the absorbance (A) was measured at 725 nm using a spectrophotometer. Gallic acid standards of different concentration (0.000, 0.0125, 0.025, 0.050, 0.075 and 0.1 mg/mL) were prepared the same way.

DPPH free radical scavenging capacity was measured using a previously reported method[66] with modification.[1] Aliquots of 1 mL 0.001% each chitosan in 0.01% acetic acid were added to 1 mL 100 µM methanolic DPPH solution. The mixture was stirred

for 30 min in dark at room temperature, followed by absorbance measurement at 517 nm. The DPPH free radical scavenging capacity was calculated using the following equation:

DPPH scavenging capacity (%) = (Abs0 – Abs1)/Abs0 × 100

where Abs0 is the absorbance of the control (DI water instead of sample) and Abs1 is the absorbance of sample.

Reducing power was determined following a reported method.[117] Aliquots of 1mL 0.025% each chitosan in 0.25% acetic acid were mixed with phosphate buffer (pH 6.6, 0.2 M) and 2.5 mL 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50 °C for 20 min followed by addition of 2.5 mL 10% trichloroacetic acid, and centrifuged 10 min at 3,315 g. Aliquots of 2.5 mL of the upper layer were added to 2.5 mL DI water, followed by addition of 0.5 mL 0.1% iron chloride solution. Absorbance of the solution was immediately measured at 700 nm.

NMR characterization of EDC/NHS activation

^{1}H, gradient HMQC- and HMBC-NMR measurements were carried out on a Varian 400-MR spectrometer equipped with a broadband probe operating at 399.78 MHz for proton and 100.54 MHz for carbon. Solid gallic acid (25 mg), EDC (29 mg) and NHS (17 mg) (1:1:1 molar ratio) were vortexed for 10 s, followed by adding 1 mL of a d_4-methanol (CD_3OD)/D_2O solution of varying concentrations (0, 25, 50, 75, 100% v/v). The resulting solution was stirred in an ice bath for 1 h. Maleic acid (1.1 M, 100 µL), as an internal standard, was added to the solution. The mixture was transferred into a 5 mm NMR tube. ^{1}H spectra were acquired with a 25 s relaxation delay, 2 scans, and an

acquisition time of 2.556 s. The FIDs (free induction decay) were transformed using Mnova (Mestrelab Research SL., Santiago de Compostela, Spain), version 10.0.1, and processed using a third order Bernstein polynomial baseline fit. All spectra were referenced to the residual D_2O signal at 4.79 ppm.

To isolate the unknown product, solid gallic acid (500 mg), EDC (580 mg) and NHS (340 mg) (1:1:1 molar ratio) were vortexed for 10 s, followed by addition of 20 mL 100% methanol. The resulting solution was stirred in an ice bath for 1 h, and 20 mL DI water was added to the reaction mixture. The solution was evaporated on the rotary evaporator. Ethyl acetate (EtOAc) (20 mL x 5 times) was used to extract GA, GA-NHS ester, and unknown product from the aqueous layer, and the EtOAc layer was washed by DI water (20 mL x 5 times). The final EtOAc fraction was concentrated to 10 mL and the unknown product, which appears on the top of GA spot on thin layer chromatography (TLC) was isolated using preparative layer chromatography on Si gel (2 mm plates, Analtech, Inc. Newark, DE) and toluene/ethyl acetate/formic acid/methanol (3:3:0.8:0.2 v/v/v/v)[118] as the eluent. The product was scraped from the plate and the Si gel was washed with acetone. Solvent removal on the rotary evaporator gave a 50 mg single product, which was analyzed by NMR.

Gradient HSQC and HMBC were acquired on the isolated sample dissolved in d_6-acetone. The HSQC experiment used 128 increments and 8 scans/increment in the F1 direction, giving a spectrum size of 962 x 128. A 90^o pulse with a relaxation delay of 1 s, an acquisition time of 0.15 s, and a one bond C-H coupling constant of 146 Hz were employed. Nonuniform sampling (NUS)[119, 120] was employed to shorten the total experimental time. The gHMBC experiment using 200 increments and 4

scans/increment in the F1 direction. A 90° pulse with a relaxation delay of 1 s, an acquisition time of 0.15 s, and the multiple-bond C-H coupling constant of 8 Hz were employed. Runs were carried out at 25°C without spinning and typically required about 10 min 47 s for gHSQC and 11 min 11 s for gHMBC. The FIDs were transformed using Mnova, version 10.0.1, and processed using a third order Bernstein polynomial baseline fit. All spectra were referenced to the residual acetone-d_6 signal at 2.05/206.26 ppm.

Statistical Analysis

All wet chemical analyses were done in triplicate. Tukey HSD (honestly significant difference test) comparison of means ($p < 0.05$) was performed using SAS (SAS Enterprise Guide 6_1, SAS Institute).

Results and discussion

The effect of solvent composition on the efficiency of grafting GA onto chitosan and on the solubility of the grafted chitosan was determined by performing the reaction in 0, 25, 50 and 75% v/v aq. EtOH. Prior to the analyses, grafting was confirmed by FTIR (Figure 2.1). Peaks at 1645 cm^{-1} and 1550 cm^{-1} correspond to the C=O stretching in amide linkages and the asymmetric bending of the free $–NH_2$ in chitosan, respectively.[121, 122] The reduced intensity of 1550 cm^{-1} peak relative to 1645 cm^{-1} peak in grafted chitosan compared to relative intensity of these peaks in non-grafted chitosan was consistent with grafting by amidation between chitosan amino groups and GA carboxyl groups. The grafted chitosan also showed a new absorption band at 1715 cm^{-1}. This peak has been assigned to the C=O stretching vibration of the ester group,[122, 123]

and in grafted chitosan resulted from the esterification between the hydroxyl groups on chitosan and carboxyl group on GA. The 1715 cm^{-1} peak intensity was higher in chitosans grafted with less EtOH indicating the possibility that grafting in pure DI water or at lower concentration of EtOH favored esterification between GA and chitosan, whereas higher concentrations of EtOH in solution favored grafting by amidation.

The effect of grafting reaction time on the grafting efficiency is shown in Figure 2.2. The efficiency of grafting in 25% EtOH achieved after 2 h was 169.20 mg GA eq/g and increased to 224.41 mg GA eq/g when the reaction lasted 6 h. However, prolonged grafting time, 12 h and 24 h, apparently did not further increase the efficiency and resulted in 197.64 and 162.63 mg GA eq/g, respectively. The lower values for grafting efficiency obtained with prolonged time may be the result of extensive cross-linking of grafted chitosan caused by the EDC and/or NHS. This, in turn, reduced solubility of grafted chitosan and consequently prevented reaction of the grafted GA with the Folin-Ciocalteau reagent, resulting in an underestimated grafting efficiency. According to Chiou and Wu,[108] the hydroxyl groups of chitosan can be activated by EDC and coupled to the amino groups, easily leading to crosslinking of the polymer. To avoid the reduced solubility and provide high grafting efficiency, a 6-hr grafting time was used for the rest of the study.

To ensure that grafted chitosan was free of residual non-grafted GA, phenolic content was determined in seven 50 mL aliquots of 75% aq. EtOH wash solution. As shown in Figure 2.3, the remaining non-grafted GA content in the spent grafting solution was 9.46 mg GA eq/mL. After the third wash, the GA content decreased to 0.104 mg GA

eq/mL, and after the fourth wash was less than 0.065 mg GA eq/mL. This confirmed that practically no free GA was left in grafted chitosans.

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The grafting efficiency was determined as Folin's total phenolics and expressed as mg GA eq per 1 g freeze-dried grafted chitosan (Figure 2.4A). The highest efficiency of 285.9 mg GA eq/g was achieved in pure DI water but decreased as the EtOH concentration in grafting solution increased. Thus, when the grafting solvent was 25% EtOH, the efficiency was 260.9 mg GA eq/g, and with 75% EtOH, the efficiency was down to 122.2 mg GA eq/g grafted chitosan. Applying the same reaction but in either pure EtOH or aq. EtOH, grafting efficiencies reported by other research groups were in the range of 65 - 209.9 mg GA eq/g.[16, 29, 30] The grafting efficacy of 285.9 mg GA eq/g achieved in our study may be due to the higher level of EDC and NHS we used, which consequently activated more GA, but also due to interferences of EtOH with the grafting reaction that resulted in lower yield found in literature.

The grafting efficiency was further indirectly assessed by determining the AOX properties as DPPH scavenging activity and as reducing power (Figure 2.4B, C). Antioxidant activity of grafted chitosan was directly related to the amount of GA grafted. Both DPPH scavenging activity and reducing power were the highest in

chitosan grafted in pure DI water (60% for 0.001% chitosan, and 0.92 for 0.025% chitosan, respectively) and, as expected, decreased with increased EtOH concentration in reaction solvent (down to 33% and 0.40, respectively).

The recovery of grafted chitosan was also affected by composition of the solvent used for grafting. When DI water was used as grafting solvent, GA-grafted chitosan formed a stable colloidal dispersion (Figure 2.5), and was difficult to separate by centrifuging. To recover grafted chitosan, 187 mL 95% EtOH had to be stirred into the 50 mL reaction mixture for 30 min, followed by cooling in the refrigerator for 30 min to precipitate chitosan. Although grafting in water had the highest grafting efficiency, the precipitation of grafted chitosan was time-consuming and considered impractical for routine grafting. As the concentration of EtOH in grafting solution increased, separation of grafted chitosan was easier. In 25% aq. EtOH, grafted chitosan was just slightly dispersed, and in 50% and 75% aq. EtOH was completely precipitated. No chitosan was dissolved (nor dispersed) in any of the solvents at the beginning of the grafting process, when all compounds were just mixed, but became dispersed in water as the reaction developed. Although chitosan dissolves in aqueous solutions only when pH is lower than 5, it extensively hydrates ("swells") in pure water. Addition of 50% or more of EtOH reduces chitosan's interaction with water and causes its precipitation.[57] Thus, when grafting is conducted in pure water, more of chitosan's active sites are available for grafting with GA. Furthermore, reactivity of GA's carboxyl group and formation of GA-NHS ester is favored in pure water compared to aq. EtOH solutions.[124] As the EtOH concentration increases to 50% or higher, it prevents hydration of the polysaccharide and thus reduces exposure and availability of chitosan's

active sites, resulting in reduced grafting efficiency and densely precipitated grafted chitosan from the grafting solution (Figure 2.5).

The solubility of freeze-dried grafted chitosan was also affected by the solvent used for grafting and by efficiency of grafting. As shown in Figure 2.6, when 0.1% freeze-dried grafted chitosan was dissolved in 1% acetic acid, chitosans grafted in pure water and in 75% EtOH had better solubility in acidified water compared to those grafted in 25% and 50% EtOH (transmittance of ~72% vs. ~53%, respectively). Good solubility of highly grafted chitosan (grafted in pure water) was most likely due to the presence of large number of bulky phenolic groups of grafted GA (1 GA at every ~3.5 glucosamine units). Additionally, water protected chitosan molecules from crosslinking by "shielding" its hydroxyl and amino groups[125, 126]. However, when a certain concentration of ethanol in the solvent was reached (approx. 25% - 50%), EDC activated the hydroxyl groups of chitosan which formed crosslinking with the amino groups[108]. On the other hand, when concentration of ethanol during the reaction was as high as 75%, it prevented hydration of chitosan molecules, EDC had limited accessibility to hydroxyl groups of chitosan, which prevented crosslinking of chitosan.

Although it is clear that presence of EtOH decreases the grafting efficiency of GA on chitosan, the factors contributing to this effect could include solubility of the polysaccharide in ethanol and competition of ethanol with chitosan's hydroxyl or amine groups for the activated carboxyl group on GA. To investigate possible causes, the effect of solvent composition on EDC/NHS activation of GA was investigated using ^{1}H qNMR. We initially investigated the coupling in d_4-methanol (CD_3OD)/D_2O solutions of a GA/EDC/NHS mixture with varying concentrations of CD_3OD, because methanol

has also been used as solvent for EDC/NHS reactions.[127] Previous reports on the structural elucidation of the reaction products between EDC/NHS and GA, (*128*) identified the singlet **a** at δ 7.04 ppm as the aromatic proton of starting GA (Figure 2.7). Similarly, peak **b** at δ 7.16 ppm was identified as expected GA-NHS ester **5.** Integration of peak **b** as a function of methanol concentration showed that as the methanol concentration increased, the amount of peak **b** produced during the reaction decreased from 33.8% to 3.4 % of the amount of original GA (stacked ^{1}H NMR spectra in Figure 2.8A). At the same time, increasing the methanol concentration resulted in the formation of a new aromatic singlet **c** at δ 6.98 ppm. As the methanol concentration was increased to 100%, peak **c** increased from 0 to 33.9% of the amount of original GA used (Figure 2.8B). To investigate the unknown GA-related product, it was isolated from the reaction mixture of GA and EDC/NHS in 100% methanol (MeOH), and characterized by gHSQC and gHMBC (Figure 2.9) We have identified this new compound as methyl gallate using 2D NMR measurements. gHSQC shows an expected one-bond correlation between C2/C6 (108.96 ppm) and their attached protons at 7.12 ppm (Figure 2.9A). Further, the one bond correlation between C8 and H8 at 51.01/3.78 is consistent with the presence of a methoxy group in the unknown product. When two bond correlations from gHMBC measurements are examined (Figure 2.9B), an additional correlation at 166.35/3.78 is observed (denoted by ¢), resulting from coupling between H8 and carbonyl carbon 7. The gHMBC spectrum also shows the expected two and three bond correlations between the H2,6 and the other carbons of the aromatic ring at 109.36/7.12 (H2/C6, H6/C2), 120.78/7.12 (H2,6/C4), 137.85/7.12 (H2,6/C1), 145.16/7.12 (H2,6/C3,5) and 166.35/7.12 (H2,6/C7). Each correlation is very similar to the correlation between the GA carbons and protons[129] and together provide support for our identification of the side product formed during EDC/NHS

coupling as methyl gallate.[130] Observation of increasing amounts of methyl gallate as the concentration of methanol in the reaction increased suggests a competitive reaction between methanol and chitosan for the activated GA intermediate **5** (Scheme 2.1).

Further verification of this competitive reaction is provided when 100% MeOH was substituted by 75% aq. EtOH, normally used for grafting gallic acid onto chitosan. Under these conditions, ethyl gallate was isolated, dissolved in d_6-acetone and measured by similar gHSQC and gHMBC measurements (Figure 2.10). One-bond correlation on gHSQC spectrum (Figure 2.10A) between C2/C6 (109.38 ppm) and H2/6 was observed at 7.13 ppm. Further, the one bond correlations between C8 and H8 at 60.08/4.25, C9 and H9 at 13.68/1.13 and two bond correlations on gHMBC (Figure 2.10B) between C8 and H9 at 60.08/1.13, C9 and H8 at 13.68/4.25 are consistent with the presence of an ethyl group in the unknown product. An additional correlation at 165.77/4.25 is observed and highlighted by ¢ in Figure 2.10B, resulting from coupling between the H8 of the ethylene group and carbonyl carbon 7. Similar to methyl gallate, the gHMBC spectrum also shows the expected two and three bond correlations between the H2,6 and the other carbons of the aromatic ring at 108.86/7.12 (H2/C6, H6/C2), 121.22/7.12 (H2,6/C4), 137.71/7.12 (H2,6/C1), 145.11/7.12 (H2,6/C3,5) and 165.77/7.12 (H2,6/C7).

These results further indicate that EtOH (or MeOH) inhibits coupling of GA to chitosan, which may proceed through a competitive conversion of the intermediate O-acylisourea ester **3** to a very stable ethyl gallate (methyl gallate) instead of the target compound, a more stable but still reactive NHS ester **5**, which will further react with –OH and $–NH_2$ on chitosan. When used as the component of solvent for grafting GA to

chitosan, EtOH not only decreases the grafting efficacy by precipitating chitosan, it also acts as another nucleophile, competing with NHS to attack O-acylisourea, forming ethyl gallate, and negatively affecting the yield of GA-NHS ester and, thus decreasing the grafting efficiency of GA at high EtOH co-solvent concentrations.

Conclusions

This study clearly showed that ethanol, as a solvent for grafting of GA onto chitosan by EDC/NHS, reduces the grafting efficiency of the reaction by acting as a reactant and decreasing the yield of GA-NHS ester. Although grafting in DI water without presence of ethanol results in the highest reaction yield, it is impractical due to additional steps needed to separate grafted chitosan from the reacting mixture. Concentration of 25% EtOH in aqueous system seems the most practical due to the high grafting efficiency and easily separable grafted chitosan. Utilizing these findings, a more efficient antioxidant biodegradable packaging material can be created for controlling lipid oxidation and extending shelf life of packaged food.

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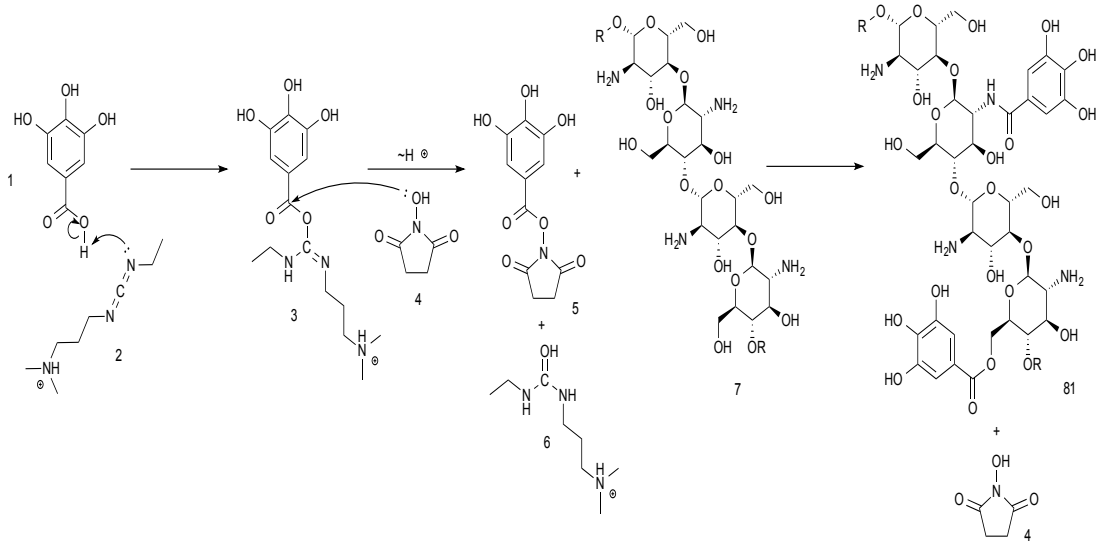
Appendix: Chapter II

Scheme 2.1 Proposed reaction pathway of grafting GA to chitosan via EDC/NHS



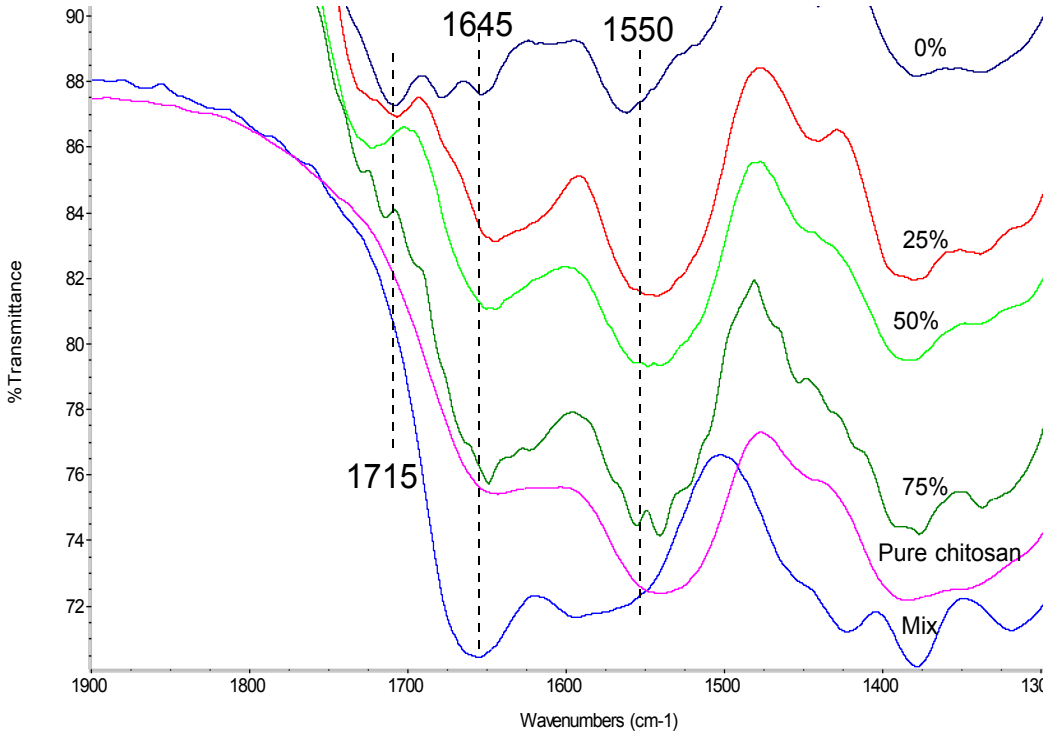


Figure 2.1 FTIR spectra of GA grafted chitosan produced in pure dionized water (black), 25% (red), 50% (green), 75% aq. EtOH (dark green), pure chitosan (pink), and chitosan mixed with gallic acid (Mix) (blue)

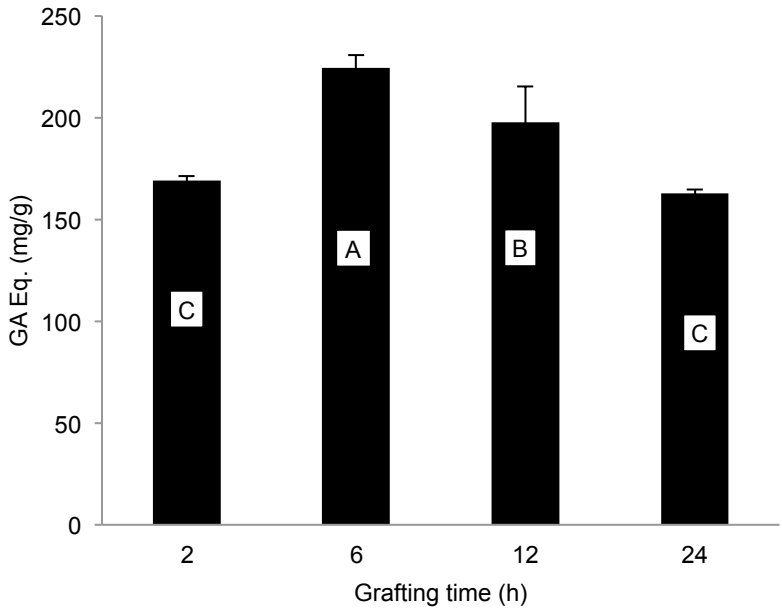


Figure 2.2 Effect of grafting time on grafting efficiency.

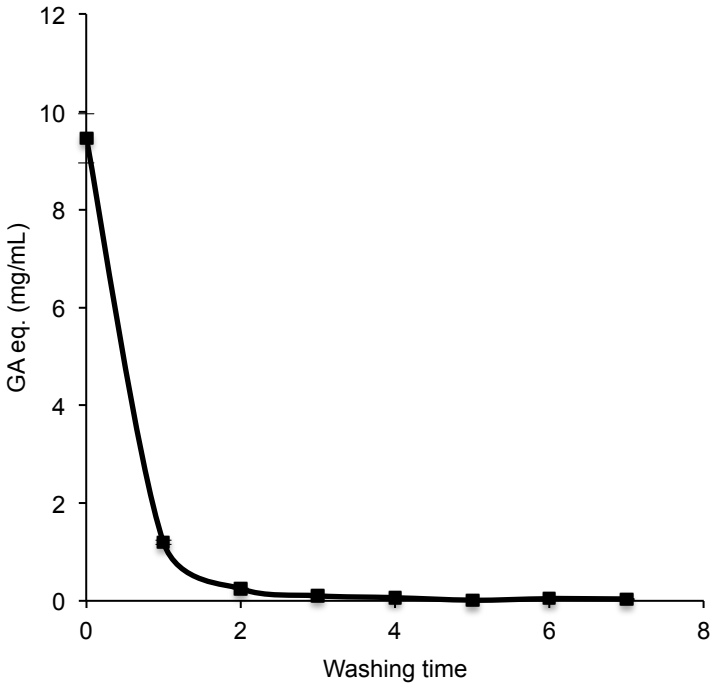


Figure 2.3 Content of free gallic acid in solution after grafting and in severn 50 mL aliquots of 75% aq. EtOH used to wash grafted chitosan (chitosan was grafted with GA in 25% aq. EtOH)

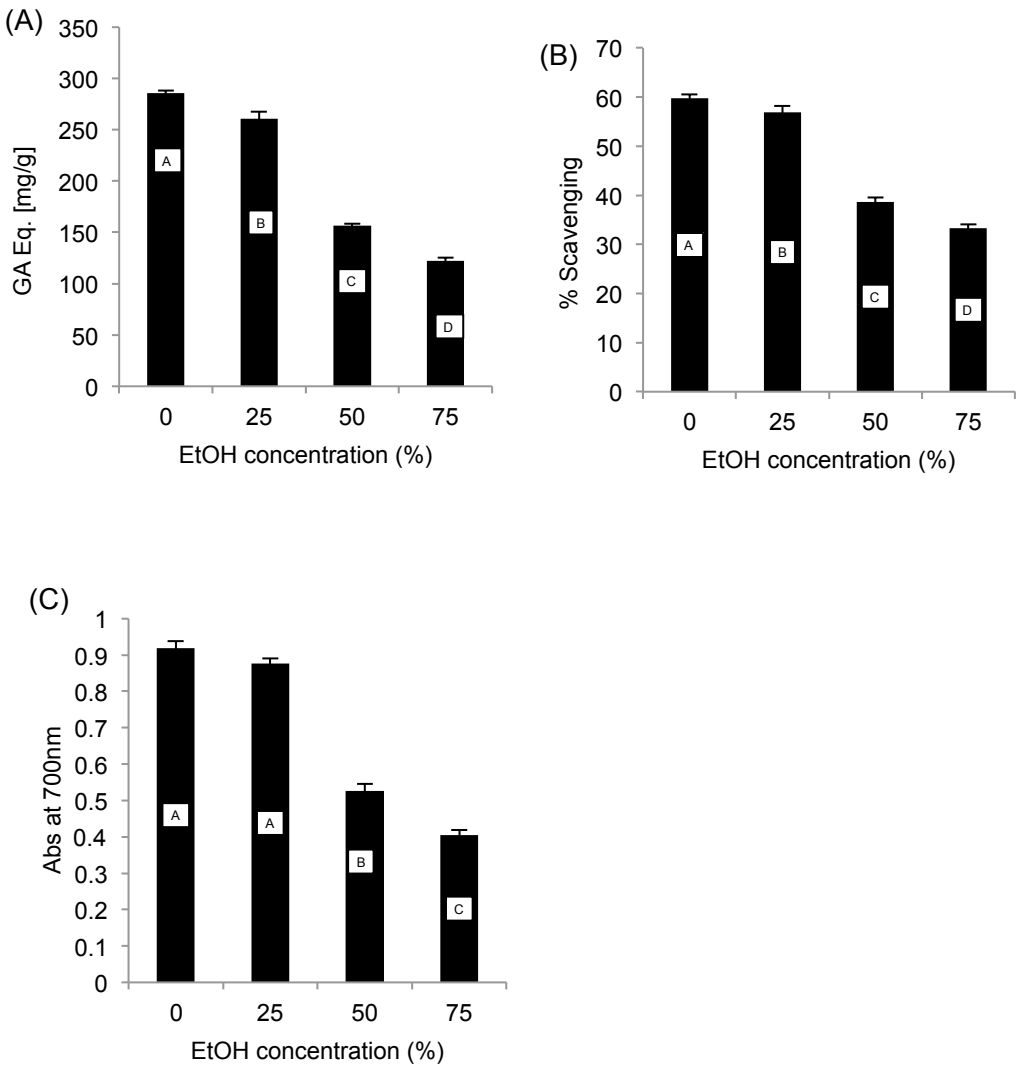
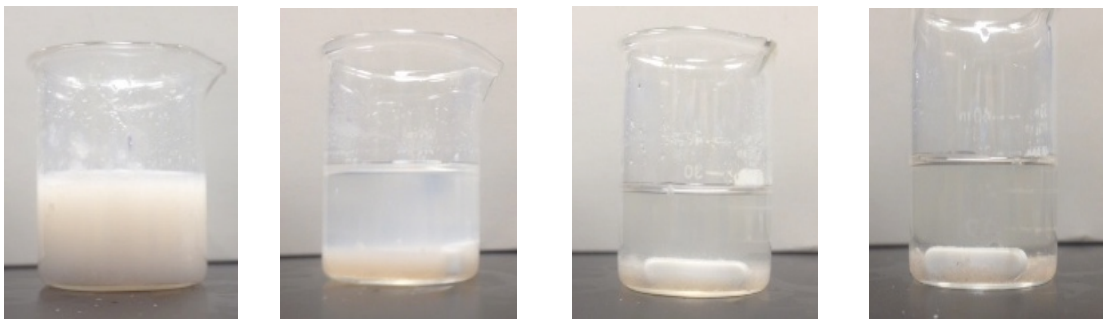


Figure 2.4 Effect of solvent composition on grafting efficiency: (A) Total phenolics (mg GA eq/g), (B) DPPH scavenging (%) (0.001% grafted chitosan in 0.01% acetic acid), (C) Reducing power (absorbance at 700nm, 0.025% grafted chitosan in 0.25% acetic acid).



0% aq. EtOH 25% aq. EtOH 50% aq. EtOH 75% aq. EtOH

Figure 2.5 Appearance of GA grafted chitosan using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) in 0% (pure DI water), 25%, 50% and 75% aq. EtOH immediately after grafting reaction.

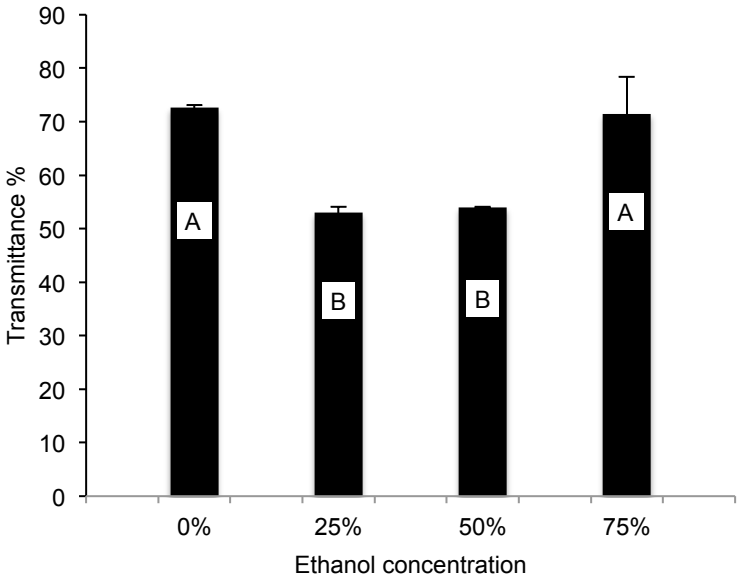


Figure 2.6 Effect of solvent composition on solubility (% transmittance at 600 nm) of grafted chitosan (0.1% chitosan dissolved in 1% acetic acid).

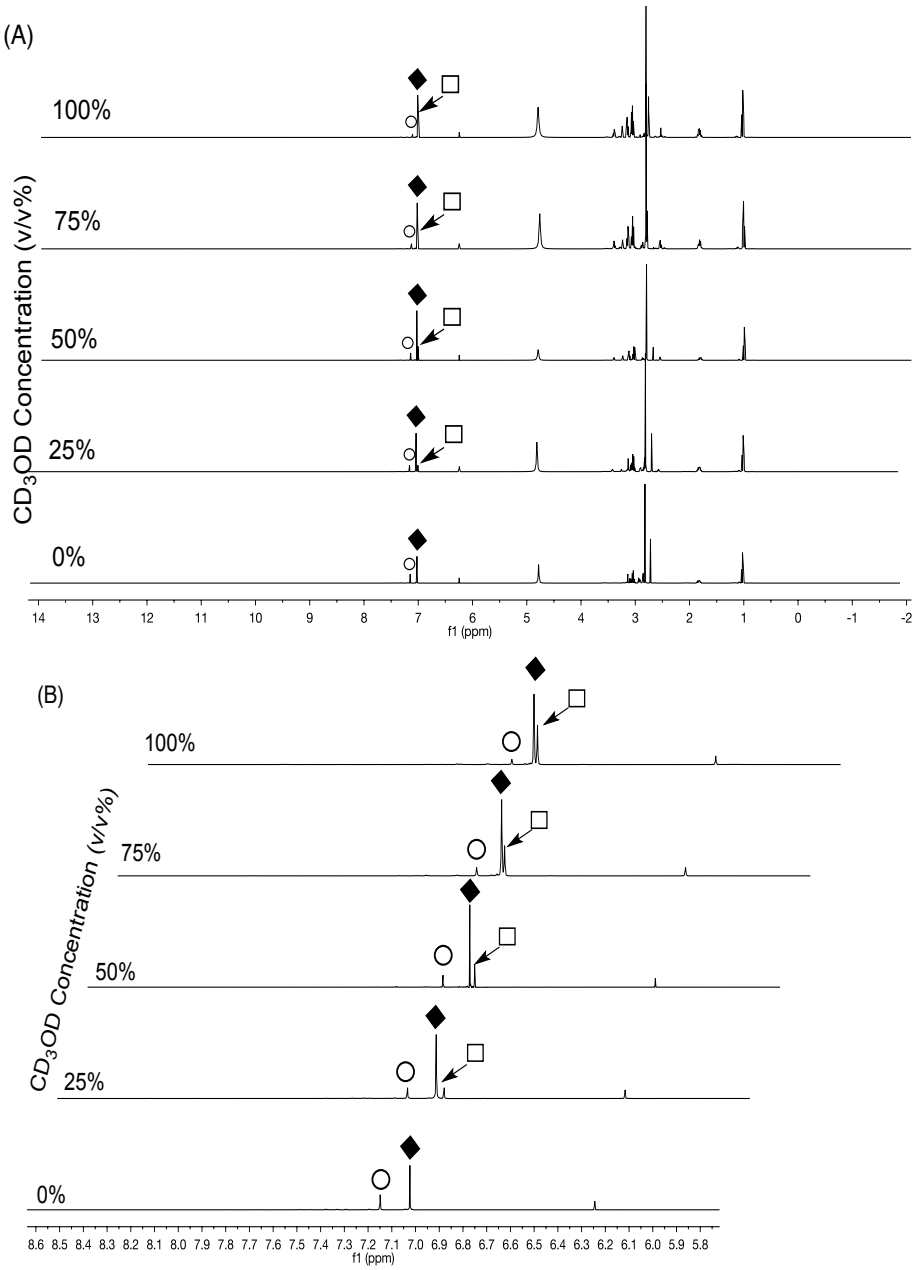


Figure 2.7 (A) 1H NMR spectra of GA, EDC and NHS reaction in d_4-Methanol (CD_3OD) /D_2O solution with different concentrations, (B) Expansion of 5.8-8.6 ppm region of the 1H NMR spectra of GA, EDC and NHS reaction in d_4-Methanol (CD_3OD) /D_2O solution with different concentrations (bottom upward: 0, 25, 50, 75, 100 % CD_3OD/D_2O). u denotes peak of GA, ¢ denotes peak of GA-NHS ester, □ denotes peak of unknown compound.

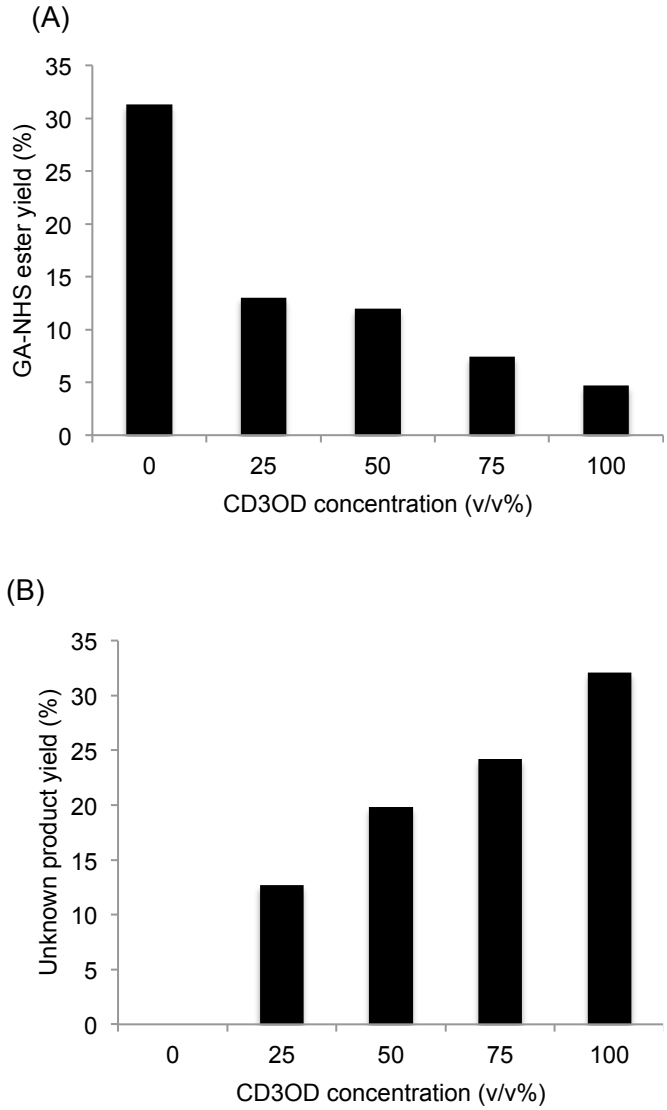


Figure 2.8 Yield (%) of GA-NHS ester (A) and GA-Ethyl ester (B) in 0, 25, 50, 75, 100 % DC_3OD.

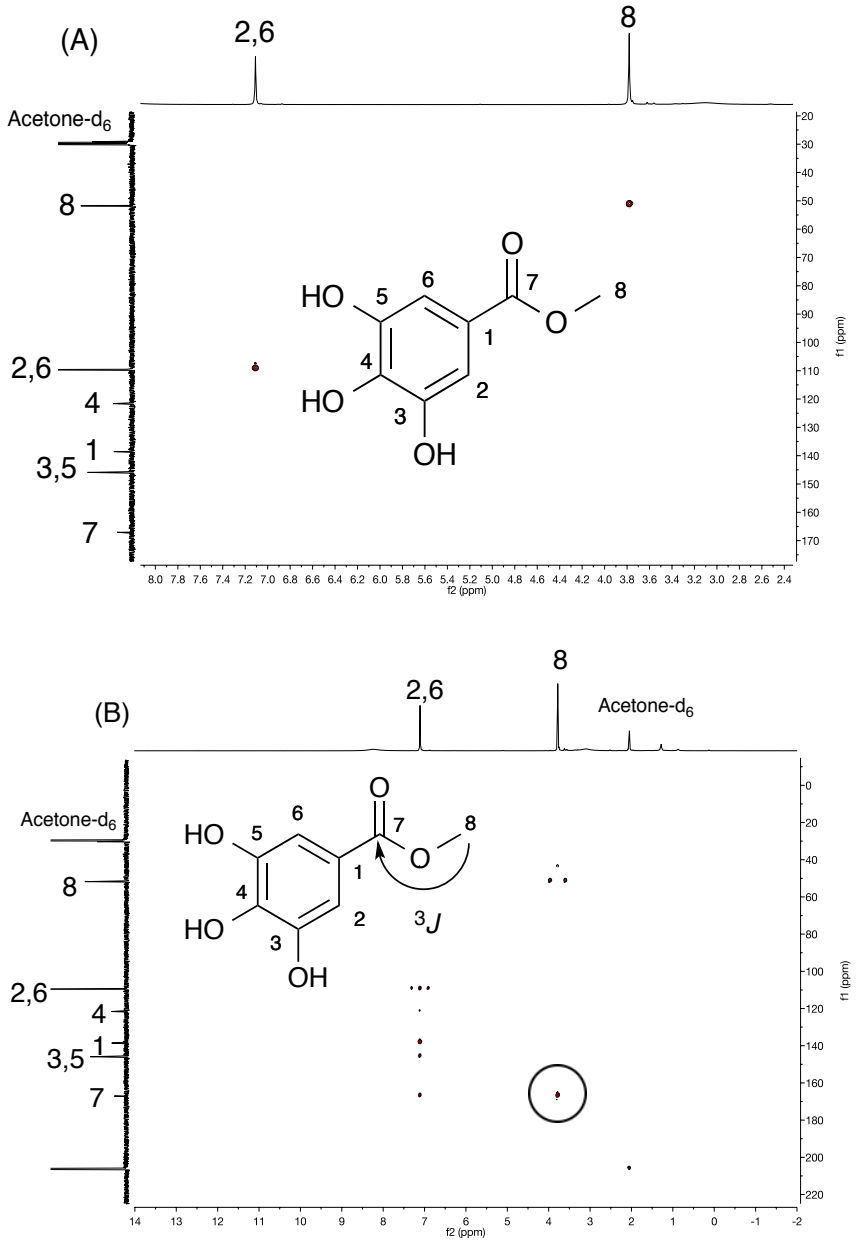


Figure 2.9 (A) gHSQC and (B) gHMBC of the isolation from reaction of gallic acid and EDC/NHS in 100% methanol. gHSQC was acquired as 128 increments and 8 scans per increment, giving an overall acquisition time of 10 min 47 s using nonuniformed sampling (NUS). gHMBC was acquired as 200 increments and 8 scans per increment, giving an overall acquisition time of 11 min 11 s using NUS.

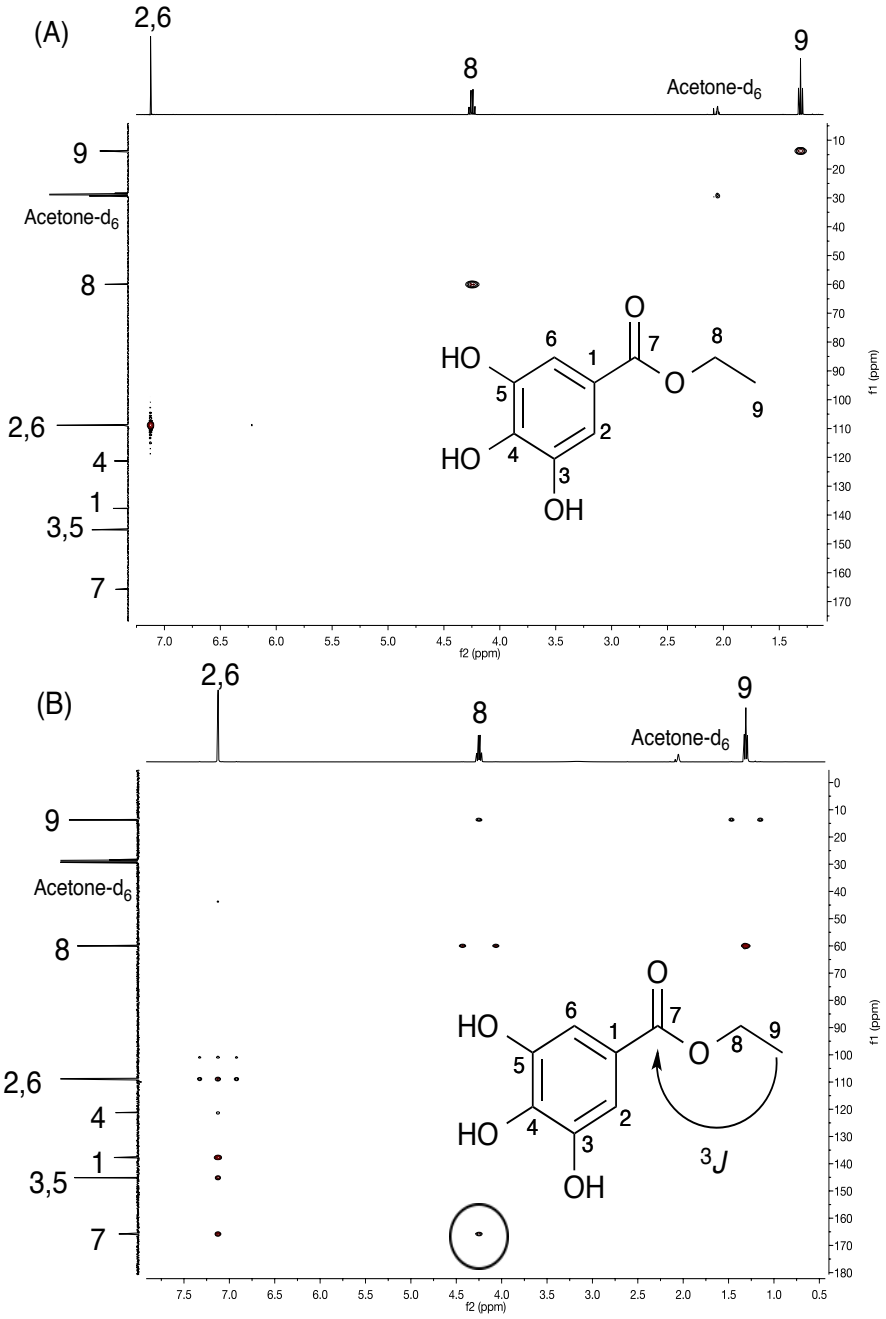


Figure 2.10 (A) gHSQC and (B) gHMBC of the isolation from reaction of gallic acid and EDC/NHS in 75% ethanol aqueous solution. gHSQC was acquired as 128 increments and 8 scans per increment, giving an overall acquisition time of 10 min 47 s using nonuniformed sampling (NUS). gHMBC was acquired as 200 increments and 8 scans per increment, giving an overall acquisition time of 11 min 11 s using NUS.

CHAPTER III

NMR INVESTIGATION OF CARBODIIMIDE ACTIVATION OF GALLIC ACID: REACTION MONITORING AND LR-HSQMBC

This chapter is a lightly revised version of a paper by the same title in preparation for submission by John D. Anderson,[1] Ping Guo,[2] Joseph J. Bozell,[1] Svetlana Zivanovic [2].

In this chapter, Ping Guo was responsible for setting up reactions for NMR experiments, acquiring NMR data and writing the introduction and part of the results and discussion in the manuscript.

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Abstract

Gallic acid (GA) can be activated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) to form GA-NHS ester. This ester has been used as an intermediate to graft GA, a strong antioxidant, to chitosan, a biodegradable non-toxic polymer, increasing the antioxidant properties of the polymer. While this grafting method has been widely used, it suffers from low efficiency, and the cause of this low efficiency remains unclear. Additionally, the evidence of the activation of GA by EDC/NHS is rarely reported. Therefore, in this study the formation of GA-NHS ester was investigated by NMR spectroscopy. To confirm the structure of GA-NHS ester, a recently developed long-range heteronuclear correlation experiment, LR-HSQMBC, was used to visualize a $^{5}J_{CH}$. From the reaction monitoring data we determined that the formation of the GA-NHS ester reaches completion at 1 h with a yield of 32.7%. By the comparison of coupling GA-NHS ester to hydroxyl group of ethanol, as a chitosan model, at 25 °C and 50 °C, we were able to prove that GA-NHS ester is stable at room temperature.

Introduction

Recently, active antioxidant packaging has received more attention from consumers and the food industry.[109, 110, 131, 132] By incorporating antioxidants into packaging material, the oxidation and spoilage of the food can be reduced.[62, 109, 111] Grafting of natural antioxidants to biodegradable packaging material is a relatively new and promising approach to address both the problems of food preservation and environmental pollution.[3, 131]

This antioxidant biodegradable packaging can be made by grafting antioxidants to biopolymers used for packaging (e.g. chitosan).[1, 33, 34, 55, 133] Chitosan is a polysaccharide of glucosamine and acetylglucosamine obtained by N-deacetylation of chitin.[24] Chitin, the supporting material of crustaceans and insects, and found in the cell walls of fungi and yeast, is the second most abundant biopolymer after cellulose. Accordingly, chitosan has been widely used in the biomedical, chemical, cosmetics, and food industries.[13, 134, 135] Modification of chitosan can be accomplished using a variety of functional compounds, via enzyme-mediated methods,[32, 38, 49] radiation cross-linking,[17, 136] physical interactions,[15] and chemical methods such as carbodiimide coupling.[1, 25, 34] Compared with the other methods, chemical modification of chitosan is advantageous because it maintains the physical, biological, and chemical activities (e.g. film forming property) and introduces new or improved properties[21] (e.g. antioxidant property)

Gallic acid (3,4,5-trihydroxy benzoic acid) is a natural phenolic compound extracted from green tea, fruits, and vegetables.[71] GA has shown significant antioxidant,

chelating, antimicrobial, and anticarcinogenic properties.[137-139] The antioxidant activity of GA, acting through rapid donation of hydrogen atoms to terminate free radicals,[137] is higher than caffeic acid, ferulic acid, Vitamin C, and Vitamin E, which are also often used as antioxidants.[3, 32, 71, 139] This makes GA an ideal antioxidant for coupling to chitosan to form antioxidant packaging material.

One chemical-mediated method for grafting GA to chitosan is through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS).[1, 33, 34, 133] EDC and NHS have been widely used to modify proteins, nucleic acids, and both natural and synthetic polymers for drug delivery, gene therapy, and improved food packaging.[40, 140-144] The mechanism of EDC/NHS activation of GA is shown in Scheme 3.1.[33] The reaction begins (scheme 3.1) with protonation of EDC **(2)**, followed by subsequent attack of the carboxylate anion **(1)** to form the O-acylisourea **(4)**, which can react with amine groups to form amide bonds or hydroxyl groups to form ester bonds. This intermediate can also react with water to form an isourea and regenerate GA.[145, 146] With the presence of NHS **(5)** in solution, the formation of GA-NHS ester **(6)** can occur, forming a more stable intermediate with a half life of hours instead of seconds.[146]

Even as ubiquitous as carbodiimide coupling is in the literature, the overall efficiency of the reaction is still low.[52, 53] According to previous studies, the efficiency of EDC/NHS activation is limited[52, 53] with a yield of NHS ester less than 25%.[52, 147] This lack of efficiency further results in the low efficiency for the grafting of GA to chitosan. Pansanphan et al. [33, 47] reported a 15% degree of substitution for the yield of GA grafting to chitosan. Although carbodiimide chemistry has been widely applied,

less emphasis has been placed on addressing the low efficiency of carbodiimide/NHS activation of carboxylic acids.[53] Recent research has indicated the complexity of EDC/NHS activation of aliphatic carboxylic acids, by showing different possible paths to form the NHS-ester,[48, 53] however, the spectroscopic evidence of the NHS ester of benzoic acids is often omitted. To gain insight into the efficiency of GA activation, reaction monitoring by ^{1}H NMR was used to investigate the formation of the GA-NHS ester, while the structures of the reactant and product molecules were determined with a standard NMR data set, including ^{1}H, ^{13}C, gHSQC, gHMBC and the recently developed LR-HSQMBC.[102]

Reaction monitoring by NMR is a powerful tool for mechanistic elucidation of chemical reactions in engineering, chemical, and biocatalyzed processes.[96-100] The analytical technique provides information not only on the concentration of each component in the reaction mixture, but also confirms the presence of various intermediates and side products forming and disappearing during the reaction.[96, 101] In this work reaction monitoring by time-array ^{1}H NMR was used to confirm the formation of a GA-NHS ester formed from the reaction of GA with EDC/NHS in D_2O.

It is challenging to confirm the expected structure of the GA-NHS ester **(6)** in Scheme 3.1: the ester **(6)** suffers from an inherently low ratio of protons to heavy atoms (e.g. C, O, N). First attributed to Phillip Crews, the Crews rule states that any molecule with a proton to heavy atom ratio less than 2 might be difficult to determine by a standard 2D NMR data set (e.g. COSY, HSQC/HMQC, HMBC, and NOESY/ROESY).[148-150] Because that the long-range coupling information (i.e. $^{n}J_{CH} > 3$) is needed to confirm the connectivity of the molecule, which a normal 2D NMR data set is not adequate for

obtaining. To confirm the structure of GA-NHS ester, a $^{5}J_{CH}$, $^{6}J_{CH}$ or $^{5}J_{CC}$ has to be visualized. Therefore a recently developed long-range heteronuclear single quantum multiple bond correlation (LR-HSQMBC) experiment[102] which can acquire $^{n}J_{CH}$ (n = 4, 5, 6) was applied to the structure elucidation of GA-NHS ester.

To further understand the effect of temperature on the reactivity of GA-NHS ester, ethanol (EtOH) was chosen as a model of chitosan to react with GA-NHS ester at different temperature (25 °C and 50 °C).

Experimental

Materials and Equipment

Gallic acid and all the solvents for extraction or isolation were purchased from Sigma–Aldrich. EDC and NHS were purchased from Acros Organics. All NMR solvents were purchased from Cambridge Isotope laboratories.

All NMR data were acquired using a Varian 400 MHz 400MR NMR spectrometer. Reaction monitoring and material characterization were carried out in 5mm 528-PP Wilmad NMR tubes with the sample temperature maintained at 25 °C. ^{1}H, ^{13}C, gHSQC, gHMBC, and LR-HSQMCB NMR data were processed using Mnova (Mestrelab Research SL., Santiago de Compostela, Spain), version 10.0.1. The Mnova Reaction Monitoring plugin was used to process the time-arrayed ^{1}H NMR reaction monitoring data. Concentration vs. time data was extracted from the time-arrayed 1H NMR data using the Mnova reaction monitoring plugin, and imported into Microsoft

Excel to generate the concentration vs. time graph. All spectra were referenced to the residual D_2O signal at 4.79 ppm.

^{1}H - NMR reaction monitoring of GA-NHS ester formation.

GA (25 mg, 0.15 mmol), EDC (29 mg, 0.15 mmol), and NHS (17 mg, 0.15 mmol) were vortexed in a test tube for 10 s. The solid reagents mixture was dissolved in 1 mL D_2O and vortexed for 15 s and the reaction mixture was transferred to a 5 mm NMR tube. The progress of the reaction was monitored with a time-arrayed 1H NMR experiment, using single scans, 90° pulse, and 25 s relaxation delay for a total of 28s between each spectrum acquired. A standard reaction solution, made of the same reagents and solvents used in the reaction monitoring experiment, was used to tune and shim prior to the start of the reaction monitoring experiment to decrease the time between reaction start and data acquisition to less than 2 min. After the reaction monitoring experiment, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC optimized for 146 Hz and 8 Hz respectively were both acquired as 962 × 200 data points with 8 scans and 128 t1 increment, and given an acquisition time of 32 m 33 s and 33 m 34 s by using nonuniform sampling (NUS).

Ethyl acetate extraction of EDC/NHS and GA reaction in aqueous solution.

GA (500 mg, 3 mmol), EDC (580 mg, 3 mmol), and NHS (340 mg, 3 mmol) were mixed in a beaker, followed by adding 20 mL DI water. The reaction was stirred at 25 °C for 1 h and the reaction solution was extracted with 20 mL EtOAc 5 times. The ethyl acetate solvent was removed explain how (e.g, by evaporation), yielding a mixture of GA and GA-NHS ester, which was subsequently analyzed by NMR.

^{1}H-^{13}C LR-HSQMBC experiment.

The ^{1}H-^{13}C LR-HSQMBC experiment was applied to a 30 mg mixture of GA/GA-NHS

ester in 600 μL acetone-d_6. LR-HSQMBC data were acquired as 962 × 512 points, with 64 scans per t1 increment, optimized for 3Hz, and a NUS sampling density of 50% for a total acquisition time of 13 h 43 m.

Coupling GA to EtOH.

GA (500 mg, 3 mmol), EDC (580 mg, 3 mmol), and NHS (340 mg, 3 mmol) were mixed in a beaker, followed by adding 20 mL DI water. After the reaction was stirred at 25 °C for 1 h, 20 mL ethanol was added to the solution. Then the reaction mixture was stirred at 25 °C for 12 h. For comparison, the same reaction mixture was stirred at 50 °C. Both reactions were monitored by TLC to observe the change. The mixture of GA product and GA from the reaction at 50 °C was extracted using ethyl acetate (20 mL×5 times), followed by isolation of the GA product using a CombiFlash® Rf 200, eluted with dichloromethane-acetone (starting at 100:0 in 0 min to 5 min, and then a linear gradient to 60:40 from 5 min to 8 min, flow rate: 20 ml/min, 15 mL per fraction). ^{1}H-^{13}C HSQC and HMBC were acquired for the isolated GA product as 962 × 200 data points with 8 scans/ 128 t1 increments and optimized for 146Hz and 8 Hz respectively, with a NUS sampling density of 50%.

Results and Discussion

A stacked plot of ^{1}H reaction monitoring data is shown in Figure 3.1, with an expanded aromatic region shown for clarity. This expansion shows a decrease in the aromatic peak of GA at 7.04 ppm over the 1.5 h reaction time. The GA aromatic peak **a** was assigned through the aid of a routine NMR structure elucidation data set, including ^{1}H,

^{13}C, gHSQC, and gHMBC acquired at the end of the 1.5 h. At 7.21 ppm, and about 0.17 ppm downfield from the GA aromatic peak, a new aromatic peak **c** that was gradually increasing during the 1.5 h reaction time, and reached a maximum yield of ~30% at around 1 h (Figure 3.2). This was expected to be the aromatic peak for the GA-NHS ester **6** (Scheme 3.1), but the standard structure elucidation data set was not sufficient to confirm the structure and identity of this molecule (Figure 3.3). To investigate the structure and identify this molecule, a long-range correlation experiment was necessary.[*102*]

LR-HSQMBC is necessary for the structure elucidation of the GA-NHS ester, which required either a $^{5}J_{CH}$ or $^{6}J_{CH}$ to confirm the molecule's structure. Using LR-HSQMBC, it was possible to visualize a very faint correlation from the methylene peaks of the new NHS species (peak **d**), which was identified by gHMBC (Figure 3.4 a) to the carbonyl carbon of the unknown GA species (peak **c**) in Figure 3.4 b, confirming the structure of the GA-NHS ester.

Though the primary focus of this work involved using reaction monitoring by 1H NMR to confirm the formation of the GA-NHS ester and to investigate the ester's reactivity with chitosan model compounds, the reaction monitoring data also offered several unexpected insights into the EDC/NHS chemistry involved in the reaction. Of these insights, and specific to GA, is the continual decrease in the concentration of GA, even after the GA-NHS ester reaches a maximum at 3600 s (Figure 3.2). The continual decrease in the GA concentration may be caused by the solubility of GA in water. If left for more than 1.5 h the reaction will precipitate a white solid, which after NMR analysis, was confirmed to be a mixture of predominantly GA, and to a lesser extent the

GA-NHS ester and EDC urea.

The peaks (**e**, **f**, **g**, **h**, **i**, **j**) for the EDC urea **7** continue to increase over 1.5 h, the peak **b** belonging to NHS actually increases over time. At the same time peaks **p**, **o**, **m**, **k**, **n**, and **l** exist in such a small concentration at the end of the reaction that they are not readily identifiable (Figure 3.5 and Figure 3.6). Still, the splitting patterns of peaks **p** and **o**, as well as **n** imply that they belong to two EDC species. Considering that the formation of the GA-NHS ester should be consuming NHS, and therefore lowering the NHS concentration (Scheme 3.1), this at first seems incorrect.

Less clear in their origin, are peaks **m**, **k**, and **l**, which appear to be singlets. In the reaction profile (figure 3.2), peaks **p**, **o**, **m**, **k**, and **l** all decrease exponentially. Considering the concentration change of NHS and unknown peaks **p**, **o**, **m**, **k**, **n**, and **l**, although it's likely that EDC and NHS formed an intermediate, which then reacted with GA to form GA-NHS ester, further study of this reaction is required to investigate the complexity of carbodiimide activation of GA.

Confirming the presence of the GA-NHS ester allowed us to confidently investigate synthetic applications of the molecule. Scheme 3.2 shows the attempts to couple GA to a simple chitosan model compound (EtOH). Reaction (a) of Scheme 3.2 was allowed to proceed for 72 h, but no appreciable change was observed by thin layer chromatography (TLC), indicating at room temperature the GA-NHS ester was not reactive enough to form the ethyl gallate product. Reaction (b) utilized the same chemistry to form the GA-NHS ester **(6)** in the first step, while in the second step after dilution to 50% ethanol, instead of reacting at room temperature the reaction was kept at 50 °C for 12 h. A new spot appeared directly above the GA spot on TLC. This

compound was isolated through CombiFlash chromatography and the gHMBC acquired on this compound (Figure 3.7) showed correlations between the ^{1}H quartet at δ 4.24 ppm and the carbonyl ^{13}C at δ 164.54 ppm. These correlations confirmed the product ethyl gallate.

After reacting with ethanol at 50 °C, only ~17 % of the GA-NHS ester was converted to ethyl gallate, which equates to only 5% of the starting GA being coupled to ethanol. The fact that under room temperature GA-NHS ester did not react with ethanol in a detectable amount revealed the inherent stability of the GA-NHS ester, which may cause the inefficiency of EDC/NHS grafting of GA to chitosan.

Conclusion

In this study, the structure GA-NHS ester was confirmed using the LR-HSQMBC experiment, which established a foundation for further study of EDC/NHS activation of GA such as side reaction caused by solvent (shown in Chapter II). The reaction profile of ^{1}H reaction monitoring by NMR revealed the yield of the GA-NHS ester reached the highest of 32.7% at 3600 s. The spectroscopic characterization of GA-NHS ester in our study builds the foundation of further improvement of the efficiency of carbodiimide activation of GA, which will increase the yield of grafting GA to chitosan to produce antioxidant active packaging.

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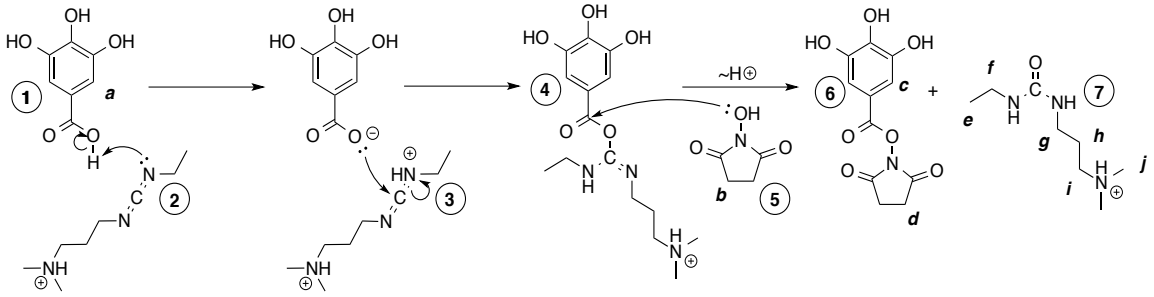
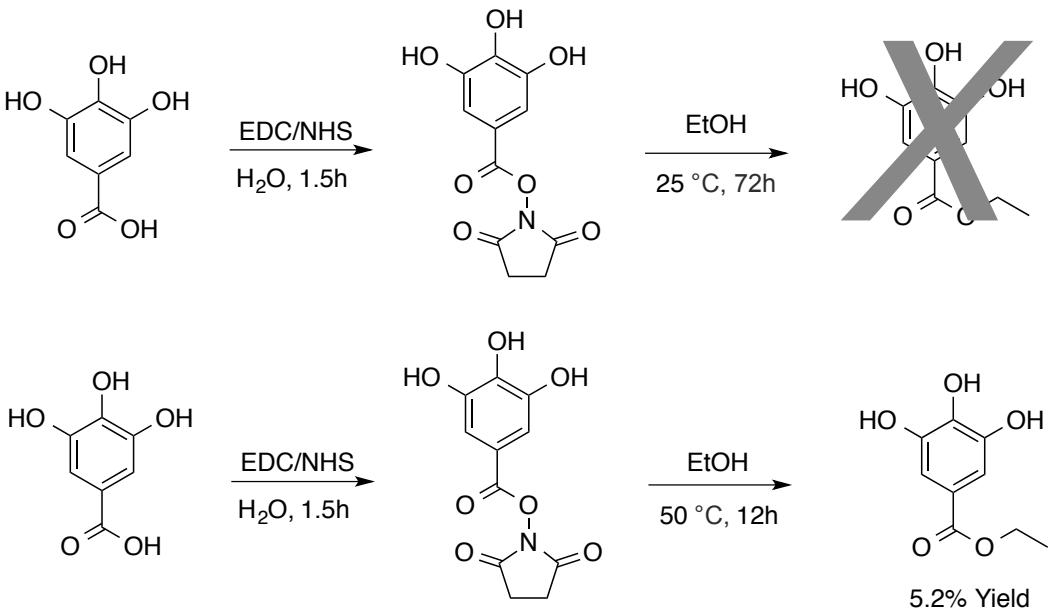
Appendix: Chapter III

Scheme 3.1 Formation of Gallic Acid - NHS Ester



Scheme 3.2 Reaction of Gallic Acid-NHS Ester with Ethanol



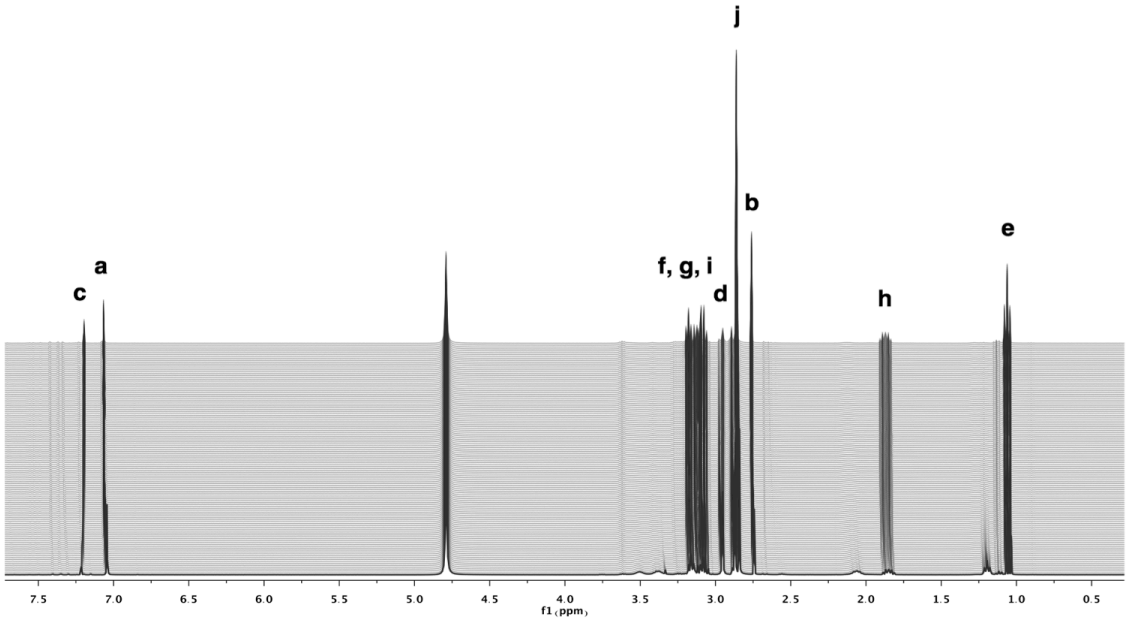


Figure 3.1 400 MHz ^{1}H time array spectra taken at 28 s intervals, single scan, and 25 s relaxation delay with a maximum of 2 minutes between reaction start and data acquisition. Reaction was setup with 1:1:1 ratio of GA 1, EDC 2, and NHS 5 in 1ml D_2O at 0.147M and 25 °C. Solid reagents mixed first, followed by D_2O.

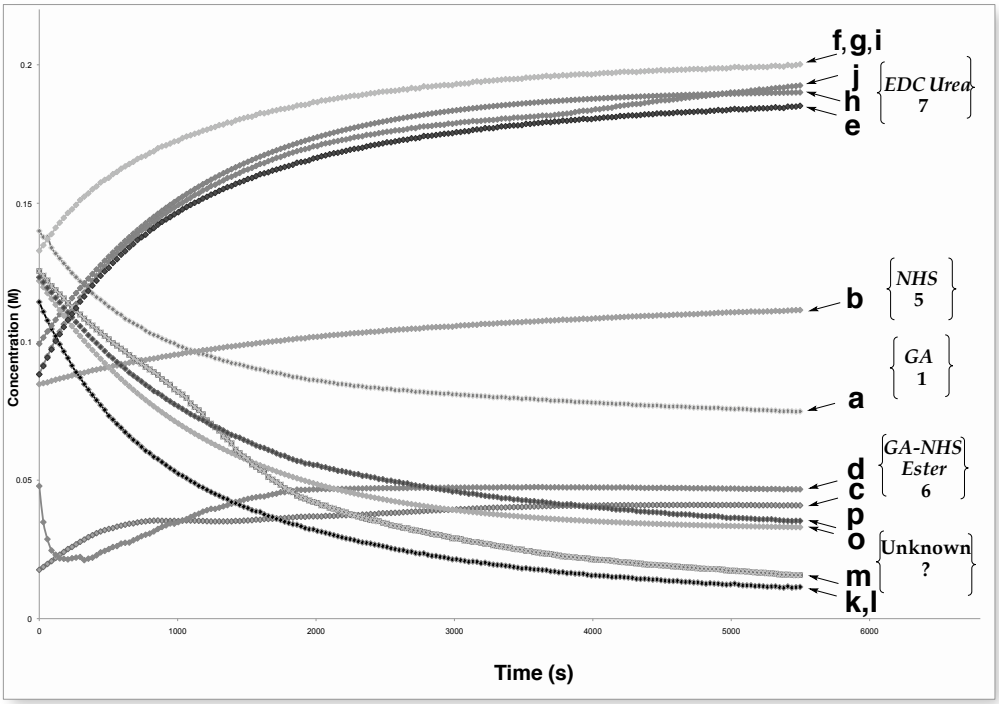


Figure 3.2 Reaction profile of ^{1}H time array reaction monitoring on reaction of GA and EDC/NHS.

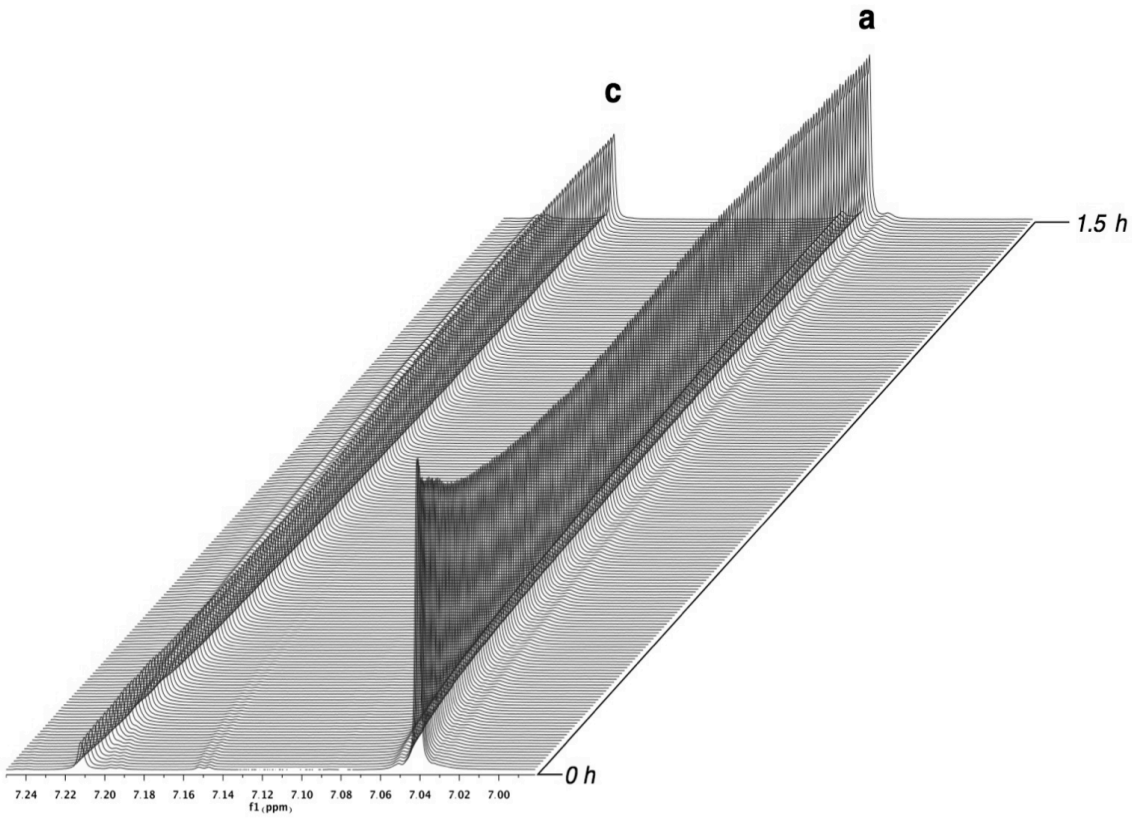


Figure 3.3 Expansion showing aromatic region, peak **a** and **c** (7.00 - 7.24 ppm).

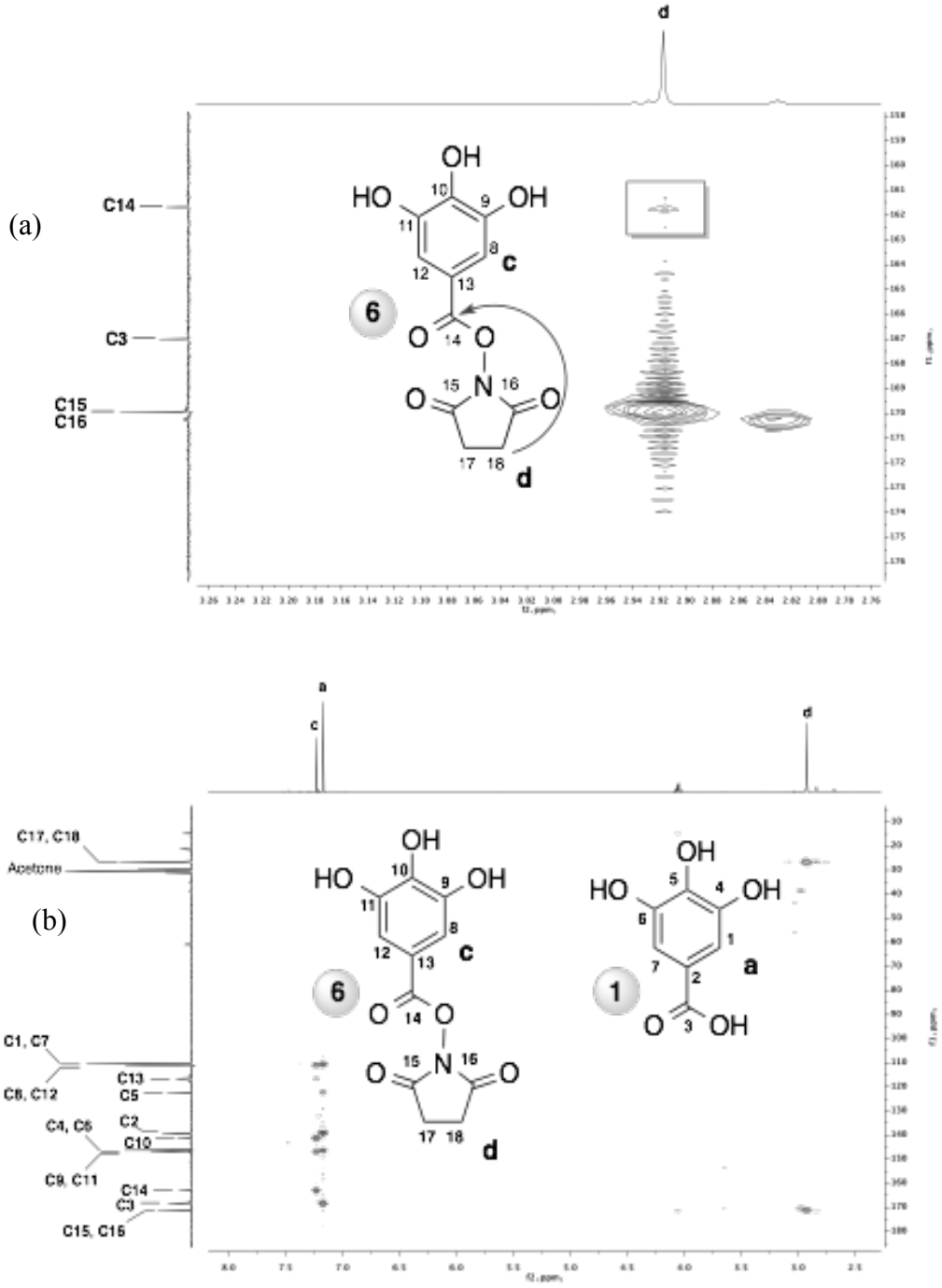


Figure 3.4 (a) ^{1}H-^{13}C gHMBC and (b) ^{1}H-^{13}C LR-HSQMBC of ethyl acetate extraction layer of reaction of GA and EDC/NHS in deionized water. (a) was acquired using 4 scans and 200 increments and optimized to 8Hz, while (b) was acquired using NUS with a sampling density of 50

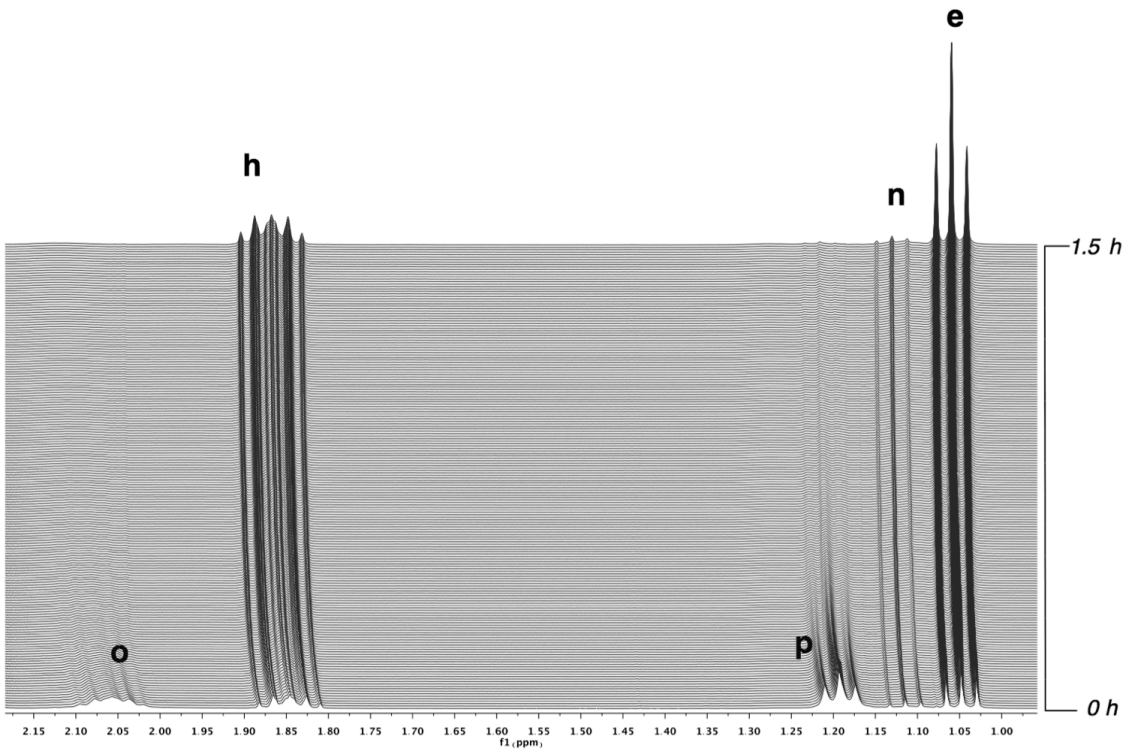


Figure 3.5 Expansion of 1H time array reaction monitoring showing EDC/NHS region (peaks **f**, **g**, **i**, **m**, **d**, **l**, **k**, **j**, and **b**).

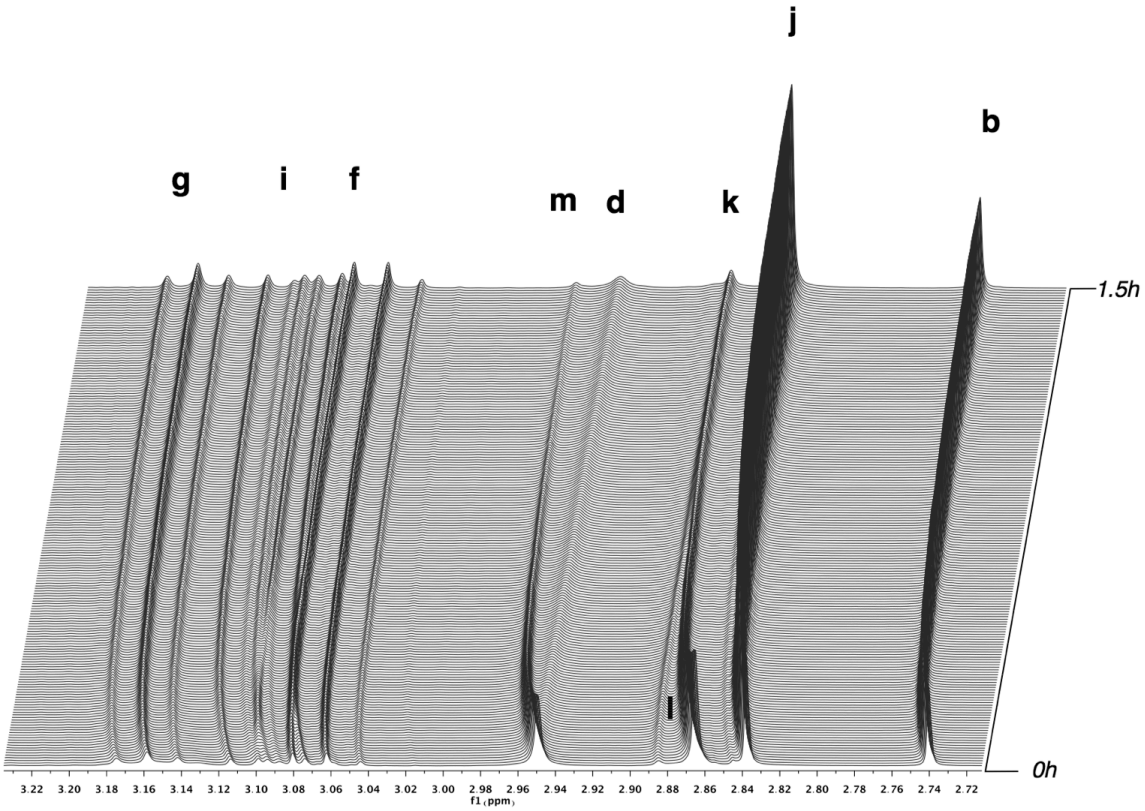


Figure 3.6 Expansion of 1H time array reaction monitoring showing low field EDC region (peaks **o**, **h**, **p**, **n**, and **e**).

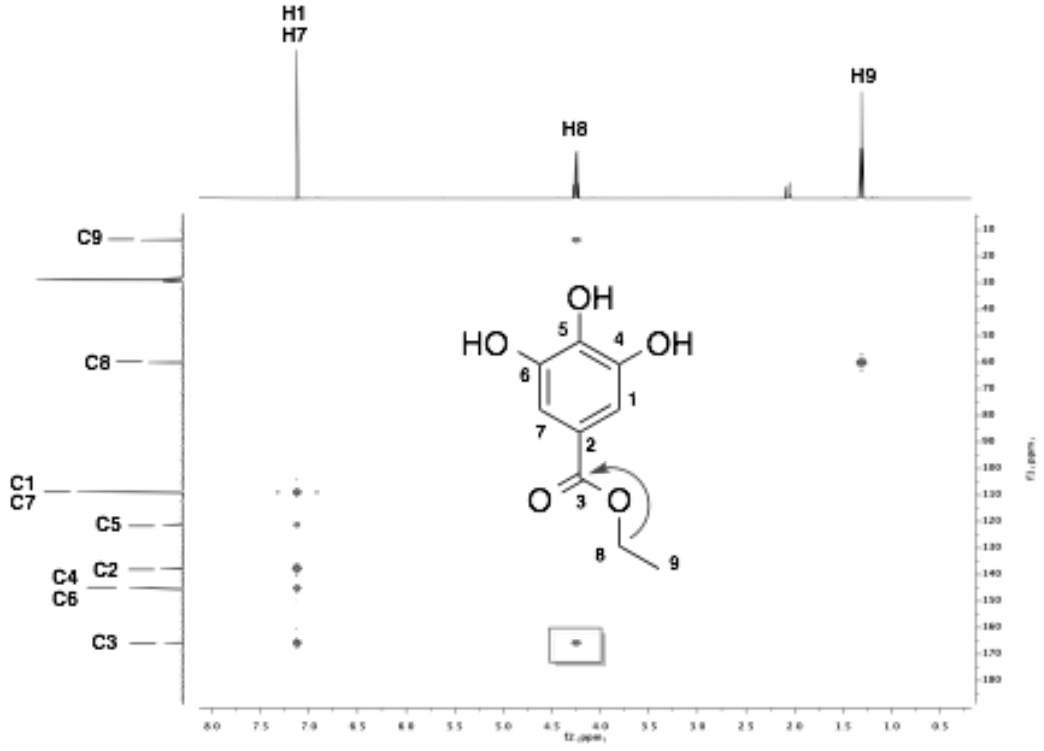


Figure 3.7 ^{1}H-^{13}C gHMBC of ethyl gallate acquired using 4 scans and 200 increments and optimized to 8Hz.

CHAPTER IV

ANTIOXIDANT PACKAGING PREPARED BY SURFACE MODIFICATION OF CHITOSAN FILMS WITH GALLIC ACID

Ping Guo was responsible for experiment design, setting up experiments, discussing with advisor Dr. Zivanovic, and writing manuscripts.

Abstract

Chitosan, as a natural polymer with a potential use in food packaging, exhibits only secondary antioxidant (AOX) activity. The primary AOX activity can be introduced by coupling of gallic acid (GA) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysuccinimide (NHS). However, extensive conjugation may decrease the solubility of chitosan, which would make it difficult to produce films from grafted chitosan powder. In this study, surface modification was introduced to chitosan films by grafting GA via esterification and amidation. Chitosan powder was grafted in the same fashion and used to produce films, while pure chitosan films served as control. FTIR-ATR analysis of the chitosan-GA films showed decrease of the NH_2 band (1550 cm^{-1}), increase of the secondary amide band (1645 cm^{-1}), and appearance of an ester band (1730 cm^{-1}) when compared to control, indicating that grafting happened at the amino and hydroxyl groups of chitosan. The AOX efficiency of modified chitosan films was assessed as DPPH scavenging activity and as reducing power using K-ferricyanide. Control chitosan films had no primary AOX activity (0% DPPH and 0.00 reducing power). The DPPH activity of surface modified films was similar to that of films from pre-grafted chitosan (75.3% and 68.6%, respectively), and reducing power of surface modified films was higher than of pre-grafted films (2.02 vs. 1.24). Surface coupling of GA to chitosan films enhanced AOX properties of the films and avoided the solubility problem caused by over conjugation of GA onto chitosan powder prior to film preparation.

Introduction

Currently, the demand for active packaging composed of biodegradable polymers and a natural antioxidant (AOX) is growing because of its potential impact on shelf life extension as well as enhanced food safety.[1, 2] Chitosan is an environmentally friendly polymer, composed of β-(1-4)-D-glucosamine and β-(1-4)-N-acetyl-D-glucosamine. It is produced by deacetylation of chitin, the second most abundant biopolymer existing in exoskeletons of crustaceans, insect, and cell walls of fungi.[3] Chitosan has been commercially applied in water and waste treatment,[4-6] cosmetics,[7, 8] food and beverages.[9, 10] The linear structure allows chitosan to form tough, flexible and transparent films.[11] Due to their antimicrobial properties, chitosan films have additional value as food packaging material.[11, 12] Furthermore, the presence of a primary amine as well as primary and secondary hydroxyl groups enable chitosan to be chemically modified.[3]

Modification of chitosan with natural AOXs from herbs and spices is attracting great interest by the scientific community because of their low toxicity.[13, 14] Thus, a number of natural AOXs have been incorporated into packaging material, including caffeic acid,[15] ferulic acid,[2] cinnamon oil,[16] and tea extracts.[17] Besides these natural AOXs, gallic acid (GA, 3,4,5-trihydroxy benzoic acid), normally present in fruits, vegetables, nuts, tea, etc[18-20] has been widely investigated for producing AOX chitosan.[21-25] As primary AOXs, GA's antioxidant activity is achieved by direct termination of free radicals by rapid donation of hydrogen atoms or electrons.[26] With three phenolic hydroxyl groups in its structure, GA exhibits strong AOX activity, while the carboxyl group enables it to be grafted to the amine and hydroxyl groups on

chitosan.[23]

Grafting GA onto chitosan has been achieved by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS).[23, 24] This method has been extensively used in amidation of proteins,[27] requires only mild reaction conditions, does not exhibit the disadvantages of the other procedures, and uses reagents (EDS and NHS) that can be easily removed after the reaction. According to published studies on grafting GA onto chitosan via EDC/NHS, the maximum grafting efficiency achieved was 65 mg GA eq/g chitosan using chitosan: GA: EDC: NHS ratio of 2×10^{-4}: 1: 0.05: 0.05 in 70% EtOH.[24] In Chapter II, we were able to increase the grafting efficiency of GA to 260.9 mg eq/g chitosan using 4×10^{-4}: 1: 1: 1 molar ratio of chitosan:GA:EDC:NHS in 25% EtOH, with complete recovery of grafted chitosan. However, the hydroxyl groups of chitosan can be activated by EDC and coupled to the amino groups, easily leading to crosslinking of the polymer[28] and reducing the solubility of the GA-grafted chitosan in aqueous acetic acid, which makes it difficult to form homogeneous film forming solutions and produce chitosan films.

In the study presented here, we aimed to develop a new route, surface modification, for the synthesis of GA-grafted chitosan films, which can be used as antioxidative biodegradable food packaging material. Surface modification of chitosan films was achieved by a two-step reaction. In the first step, GA was activated by EDC/NHS to form an intermediate, GA-NHS ester. In the second step, pure chitosan films were immersed in the solution of GA and EDC/NHS reaction mixture to allow GA-NHS ester to react with $-NH_2$ and/or –OH of chitosan molecules on the surface of the films. GA-surface-grafted chitosan films were compared with films made of GA-grafted

chitosan powder (pre-grafted) and of pure chitosan powder (control) in terms of AOX properties, GA release, physical and mechanical properties, and the ability to extend shelf life of packaged food.

Experimental

Materials and equipment

Chitosan with an average molecular weight of 307 kDa and 80% degree of deacetylation (DDA), was donated by Primex. EDC (99.8% purity) and NHS (98% purity) were purchased from Acros Organics. GA was purchased from Sigma-Aldrich.

Purification of chitosan

Chitosan flakes were dissolved in 1 wt% acetic acid to form a 1 wt% chitosan solution. The solution was stirred overnight, filtered through Miracloth®, and chitosan was precipitated by adjusting pH to ~10. The precipitate was washed with deionized water until neutral and freeze-dried. Purified chitosan was kept in a desiccator at room temperature until needed.

Synthesis of pre-GA-grafted chitosan powder

Pre-GA-grafted chitosan was prepared using a modified method from the literature.[23] GA (0.500 g, 3 mmol), EDC (0.580 g, 3 mmol) and NHS (0.340 g, 3 mmol) were mixed as solids, added to 20 mL 25% aq. EtOH, and stirred in an ice bath for 1 h. Chitosan (0.32 g, 1.18 μmol) dispersed in 30 mL 25% aq. EtOH was added to the solution, stirred for 0.5 h in ice-bath, followed by 6 h at room temperature. After the

grafting was completed, the product was centrifuged at 3,315 g for 20 min, washed 3 times with 50 mL aliquots of 75% EtOH, and freeze-dried.

Preparation of the films

All films were prepared from film forming solution (FFS) containing 0.7% chitosan dissolved in 1% acetic acid. Pre-grafted GA-chitosan films were prepared by solubilizing already grafted chitosan while control films and those prepared for surface grafting were made from purified chitosan. Portions of 10 mL and 27 mL FFS were cast in 5 and 10 cm-petri dishes, respectively. Petri dishes were left at room temperature for 4-5 days until the films were dry. When considered dry, the films were peeled off from petri dishes and kept in a fume hood for 1 month to evaporate any residual acetic acid. Smaller films were used for film characterization and larger for storage studies.

Half of the films prepared from purified chitosan were further used for surface grafting. GA (0.218 g, 1.3 mmol), EDC (0.127 g, 1.3 mmol) and NHS (0.074 g, 1.3 mmol) were mixed as solids, added to 4.4 mL 25% EtOH, and stirred in an ice bath for 1 h. This reaction mixture was then added to a petri dish with 5 cm chitosan film (0.07 g film) immersed in 6.6 mL 25% aq. EtOH. In the modification of 10 cm chitosan films for food storage, the amount of reagents and solvents was doubled. The reaction system was shaken (WU-51706-00,Thermo, Madison, WI) for 0.5 h in a walking cooler (3 $^{\circ}$C) followed by 6 h or 12 h shaking at room temperature. After the grafting was completed, the films were washed 3 times with 50 mL aliquots of 75% EtOH by shaking for 10 min each time, and dried in room temperature for 4-5 days.

Confirmation of grafting and characterization of GA-grafted chitosan

FTIR spectra were acquired from the chitosan films (CF), pre-grafted CF and surface-grafted CF between 500 and 4000 cm^{-1}, with 128 scans and resolution of 4 cm^{-1} (Nicolet NEXUS 670,Thermo, Madison, WI).

Determination of antioxidant properties of GA-grafted chitosan

DPPH free radical scavenging capacity was measured using a previously reported method[18] with modification.[24] 1 mg pre-grafted CF or surfaced-grafted CF was immersed in 1 mL 100% methanol and stirred for 1 h. Methanolic DPPH (1 mL 100 μM 2,2-diphenyl-1-picrylhydrazyl) solution was added and the mixture was stirred for 30 min in the dark at room temperature, and the absobance at 517 nm was measured. The DPPH free radical scavenging capacity was calculated using the following equation:

$$\text{DPPH scavenging capacity} = (Abs0 - Abs1)/Abs0 \times 100$$

where Abs_0 is the absorbance of the control (DI water instead of sample) and Abs_1 is the absorbance of sample.

Reducing power was determined following a reported method.[29] Pre-grafted CF or surfaced-grafted CF (1 mg) was immersed in 1 mL 100% methanol and stirred for 1 h, followed by mixing with phosphate buffer (2.5 mL, pH 6.6, 0.2 M) and 2.5 mL 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50 °C for 20 min followed by addition of trichloroacetic acid (2.5 mL 10%), and centrifuging at 3,315 g for 10 min. Aliquots of 2.5 mL of the upper layer were added to 2.5 mL DI water, followed by adding iron chloride solution (0.1%, 0.5 mL). Absorbance of the solution

was immediately measured at 700 nm.

Release test

The release test of GA from the films (pre-GA-grafted CF, surface-grafted CF and CF mixed with GA) was carried out by determining the migration of GA from the films into 95% EtOH.[30] Aliquots of 0.1 g of each sample were immersed in 40 mL 95% EtOH and stirred in room temperature. Portions of 5 mL of the solution were taken and replaced by 5 mL fresh 95% EtOH every 1 h in the first 5 h and every 5 h in the following 10 h, and used for total phenolic content measurement. Total phenolics content was determined by Folin-Ciocalteau method [31] with modification[24]. Briefly, 1 mL of each solution from the release test was added to 7 mL DI water with 1 mL Folin-Ciocalteau reagent. After 3 min, 12.4% sodium carbonate solution was added to the mixture, and the solution was vortexed. The mixture was kept at 40°C for 30 min, after which the absorbance was measured at 725 nm using a spectrophotometer. Gallic acid standards of different concentration (0.000, 0.0125, 0.025, 0.050, 0.075 and 0.1 mg/mL) were prepared the same way.

Physical characterization

Physical characterization of the films was conducted after conditioning in a desiccator at 25% relative humidity (RH) at room temperature. Thickness was measured with a hand-held microcaliper (Mitutoya Corp., Kawasaki, Kanagawa, Japan), color using a colorimeter (Hunter Lab Miniscan XE Plus, Hunter Associates Laboratory, Reston, VA) standardized with white and black tiles.

Tensile strength and elongation were measure on TA.XTplus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY). Dog-bone shaped film strips (20 mm

×18 mm of width in the two side and the middle part 5 mm × 40 mm) were held between two clamps positioned at a distance 4 cm. During measurement, the film was pulled by the top clamp at a rate of 0.5 mm/s to a distance of 5 cm before returning to the starting point. The force and elongation were measured when the films broke. Measurements were run four times for each film. The tensile strength and elongation at break were calculated as below:

Tensile strength (N/mm^2) = Breaking force (N)/Cross-sectional area of sample (mm^2)

Elongation at break (%) = Increase in length at breaking point (mm)/Original length (mm) x 100%

Water vapor permeability (WVP) was determined based on weight loss of evaporated water from a Fisher/Payne permeability cup (35 mm opening; Thermo Fisher Scientific, Waltham, MA) placed in a desiccator (RH 25%) during 24 h at 25 °C (Fisher Scientific, 1984).

Lipid oxidation of packaged sunflower seeds powder

Raw sunflower seeds (The Fresh Market, Greensboro, NC) were ground to a powder in a coffee grinder. The powder (4 g) was packaged in pouches made of non-modified CF, pre-GA-grafted CF, surface-grafted CF and polyethylene (PE, Glad, Rogers, AR). The pouches were made by attaching two 10 cm-diameter films or two 10 cm-diameter circular cutouts from PE bags using adhesive (Scotch Double Sided Tape). The pouches were placed in an environmental chamber (IG 420U, Yamato Scientific Co., Ltd.) at 50 °C and 30% RH. The packaged sunflower seed powder was analyzed after 0, 4, 12 and 20 weeks.

The lipid oxidation of the packaged powder was assessed according the Current Protocols in Food Analytical Chemistry.[32, 33] Sunflower oil was extracted by adding 12 mL hexane to 2 g of the powder and the mixture was stirred for 1 h, followed by two cycles of centrifuging. The solvent was removed from oil on a rotary evaporator (CH-9230 Flavil, Switzerland). To measure conjugated dienes (CD) and trienes (CT), 0.01 - 0.05 g oil extract was dissolved in 2,2,4-trimethylpentane (iso-octane) in 25 mL volumetric flask and followed by measuring the absorbance at 233 nm for CD and 268 for CT on a UV-Vis spectrophotometer. The results were expressed as:

$$E=A\lambda/(cL \times I),$$

where E is the extinction value, Aλ is the absorbance, cL is the concentration of the lipid solution (g/100 mL), and I is the path length of the cuvette in cm.

Peroxide value (PV) of extracted sunflower oil was determined based on the ability of the peroxides formed in lipids to oxidize ferrous ions. Oil (0.01 - 0.50 g, extracted from samples) was dissolved in 10 mL chloroform/methanol (7:3), and added to 100 µL 10 mM xylenol orange solution followed by addition of 50 µL iron (II) chloride solution. After 5 min, the absorbance at 560 nm was measured. A standard curve was constructed by iron (III)-chloride standard solution (0, 1, 2, 4 µg/mL) added to 100 µL 10 mM xylenol orange solution. PV was calculated using the flowing equation:

$$PV=[(AS - AB) \times mi]/(W \times 55.84 \times 2)$$

Where AS is the absorbance of the sample, AB is the absorbance of the blank, mi is the inverse of the standard curve slope, W is the weight of the sample (g), and 55.84 is the atomic weight of iron.

To determine the amount of thiobarbituric acid reactive substances (TBARS), 1 g of sunflower seed powder was mixed with 0.5 mL antioxidant solution (prepared by dissolving 0.5 g propyl gallate and 0.5 g ethylenediamietetraacetic acid into a 100-mL volumetric flask) and 10 mL ice-cold trichloroacetic acid reagent (20 w/v% TCA) for 2 min, followed by addition of 10 mL ice DI water. After 1 min, the mixture was filtered using a Büchner funnel with Whatman #1 filter paper, and then 0.45 μm filters. Extract (5 mL) was added to 5 mL 0.02 M thiobarbituric acid (TBA) and kept in a boiling water bath for 35 min, followed by cooling in an ice bath for 5 min. Absorbance was measured at 532 nm. Standards were prepared by adding 0.5, 1, 3, and 5 mL 0.2 nM 1,1,3,3-tetramethoxypropane in a 100 mL volume flask which was then filled with TCA reagent/water solution (1:1). TCA reagent/water solution (1:1) was used as a blank. The result was expressed as mg MDA eq/kg sunflower seed powder.

Statistical Analysis

All wet chemical analyses were done in triplicate. Tukey HSD (honestly significant difference test) comparison of means ($p < 0.05$) was performed using SAS (SAS Enterprise Guide 6_1, SAS Institute).

Results and discussion

Grafting was confirmed with FTIR (Figure 4.1). The peak at 1651 cm^{-1} is assigned to C=O stretching in amide and the peak at 1595 cm^{-1} is attributed to the asymmetric bending of the free $–NH_2$ in chitosan.[34] The reduced intensity of the 1550 cm^{-1} peak relative to the 1645 cm^{-1} peak in grafted chitosan films compared to the relative

intensity of these peaks in non-grafted chitosan was an indication of amidation between GA carboxyl groups and chitosan amino groups. The spectra of GA-surface-grafted chitosan and grafted chitosan powder also showed a new absorption band at 1715 cm^{-1}. This peak was assigned to the ν (C=O) stretching vibration of the ester group,[35, 36] resulting from the esterification between the carboxyl group on GA and the hydroxyl groups on chitosan.

The grafting efficiency was assessed by testing the AOX properties as DPPH scavenging activity and as reducing power of GA-surface-grafted chitosan films and pre-GA-grafted chitosan films (Figure 4.2). Antioxidant activity of grafted chitosan films was directly related to the amount of GA grafted. DPPH scavenging activity was similar in GA-surface-grafted chitosan films and pre-GA-grafted chitosan films, both produced by 6 h grafting (71.7% and 70.7%, respectively). Similarly, reducing power of these films was not significantly different (1.24 and 1.14, respectively). However, GA-surface-grafted chitosan films produced by 24 h grafting had stronger AOX activity. The DPPH scavenging ability and reducing power increased with the extension of grafting time for surface modification (to 89.9 % and 2.02, respectively). If grafted for the same time, surface grafting of GA had the same effect on chitosan films as grafting GA onto chitosan powder, and grafting efficiency increased with prolonged surface grafting time.

Release studies were carried out by exposure of the films developed to 95% EtOH. The concentration of GA released was determined as Folin's total phenolics and expressed as mg GA eq per 1 mL 95% EtOH (Figure 4.3). The difference in GA release from chemically grafted was compared to physically mixed in chitosan films was clearly

noticeable. The concentration of GA in 95% EtOH released from both surface-grafted and pre-grafted chitosan films was similar and slowly increased with time. The concentration of GA released from chitosan films in which it was just mixed, was high and rapidly increased, similar to previously reported data.[37, 38] According to these results, chemically grafting of GA onto biopolymer molecules has a clear advantage over just mixing it in the FFS since a lower amount of GA leached from such packaging material into food products.

GA-surface-grafted chitosan films and non-modified chitosan films had similar thicknesses of 26-27 μm, and were thinner than pre-grafted chitosan films (33 μm). As shown in Figure 4.4, chitosan films were colorless, while surface-grafted chitosan films had a slightly lower L* (L*=87.59 and b*=3.90), which may be caused by the oxidation GA grafted on the surface of the films. Films of these two types were transparent while pre-grafted films were dark yellow (L*=83.40 and b*=18.40) and cloudy (Figure 4.4). The color change of pre-grafted films was likely due to the oxidation of the grafted GA and the cloudiness may be result of partially undissolved polymers in the FFS. Tensile strength and elongation of chitosan films was 1,300 kg/cm^2 and 3.19%, which were significantly reduced by both pre-grafting and surface grafting (566, 351 kg/cm^2 and 1.13, 0.71%, respectively). This is possibly due to crosslinking of chitosan during grafting using EDC/NHS which increased the stiffness of chitosan films.[28, 39]

WVP (Table 1) was not significantly different ($p<0.05$) for three types of chitosan films, indicating that grafting GA onto chitosan did not affect this important property. WVP can be affected by different factors, e.g. with addition of a hydrophilic

compound[40] or crosslinking.[41] In the GA-grafted chitosan films, these two effects were balanced out and no significant change in water vapor transfer through the film occurred.

The effect of the packaging material on protecting sunflower seed powder from oxidation was evaluated based on formation of primary lipid oxidation products as conjugated dienes and trienes, peroxide value, and secondary lipid oxidation products as amount of TBARS (Table 2). During the first 4-week storage (Figure 4.5), the sunflower seeds packaged in the pouches made of pre-grafted chitosan films and surface-grafted chitosan films were of similar quality compared to those packaged in PE bags and the amount of TBARS was lower than in the sunflower seeds packaged in non-modified chitosan films. After 12-week storage, the sunflower seeds packaged in both grafted chitosan films had lower CT value and amount of TBARS but higher CD and PV values than those packaged in non-modified chitosan films and PE bags. This is likely as with longer storage time, the formation of secondary oxidation products and the decomposition of primary oxidation products of sunflower seeds packaged by both GA-chitosan films (pre-grafted and surface-grafted) happened slower than those packaged in PE bags and non-modified chitosan pouches.[42-44] Abreu et al.[44] reported similar results on PE films incorporated with antioxidants from barley husks compared to non-modified PE films, which confirmed that incorporation of AOX into PE films was able to slow primary and secondary oxidation. Therefore, the results of food storage test indicated that grafted chitosan films have similar effects on inhibiting lipid oxidation, but better than non-modified chitosan films and PE bags.

Conclusion

This study reported the surface grafting of chitosan films with GA using EDC/NHS. Surface modification of chitosan films was able to keep the transparency and avoid the solubility problem caused by crosslinking between chitosan molecules initiated by grafting reagent EDC. On the contrary, it decreased the color change caused by GA oxidation and does not alter water vapor barrier properties of pure chitosan films. Superior antioxidant properties can be achieved with grafting of GA onto the surface of chitosan films for prolonged time. Grafting of GA on chitosan inhibited the leaching of GA into food products and could effectively prevent lipid oxidation in packaged sunflower seeds. Thus, the active packaging film prepared by surface grafting chitosan films with GA via EDC/NHS may be a potential packaging material to maximize the prevention of lipid oxidation in different food model systems.

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Appendix: Chapter IV

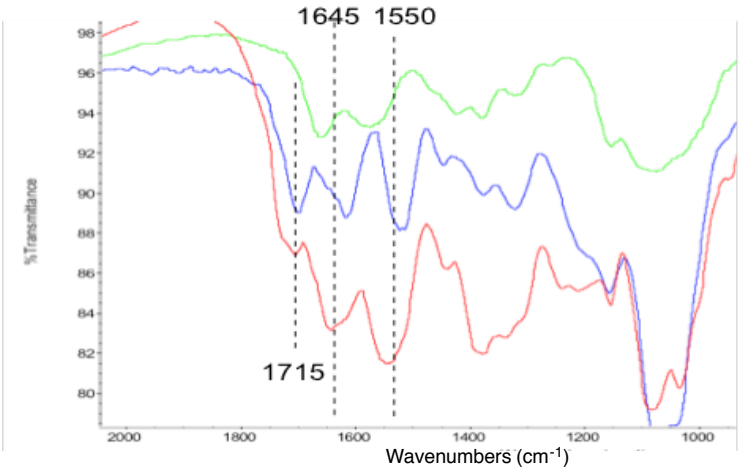


Figure 4.1 FTIR spectra of non-modified chitosan (green), gallic aicd-surface-grafted chitosan film (blue), gallic acid-grafted chitosan powder (red).

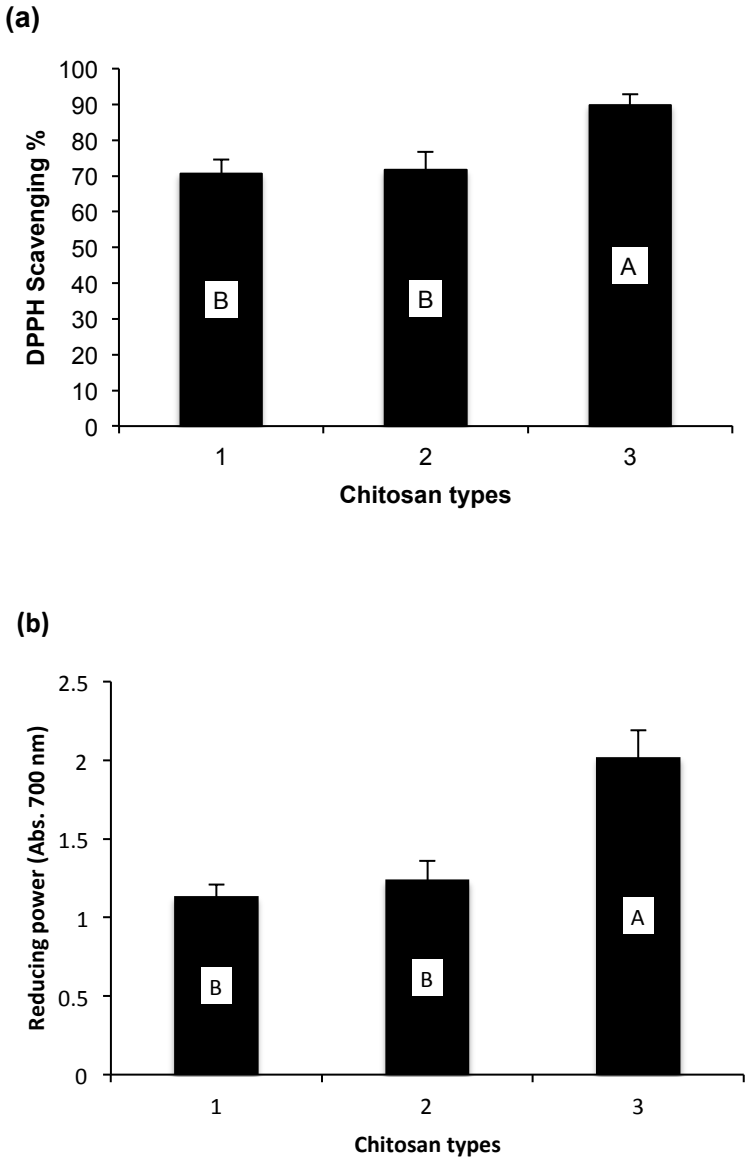


Figure 4.2 DPPH Scavenging (%) (a), and Reducing Power (absorbance at 700nm) (b) chitosan powder grafted for 6 h (1), chitosan films grafted for 6 h (2), 24 h (3) and values are presented as means with standard deviation. Bars with different letters are significantly different ($p < 0.05$).

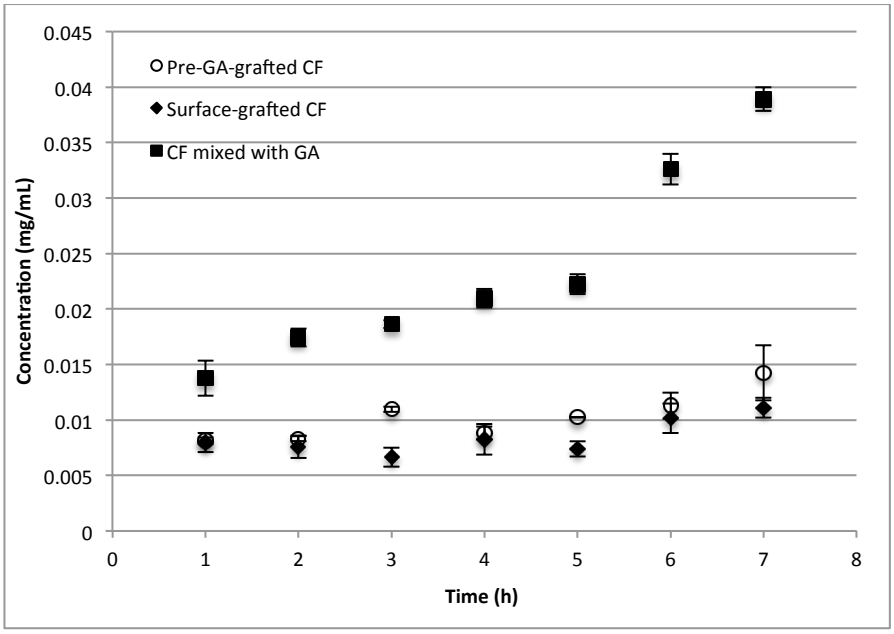
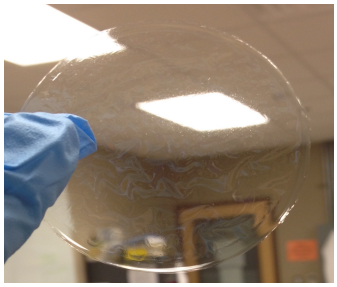
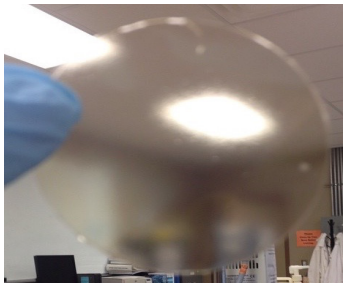
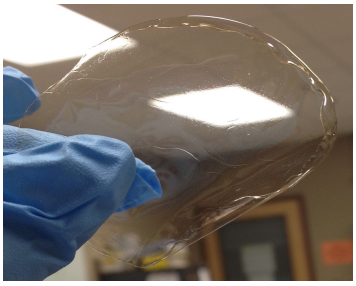


Figure 4.3 Release of gallic acid from pre-gallic acid-grafted chitosan films (¢), Surfaced-grafted chitosan films (u), and chitosan films mixed with gallic acid (n) into fatty food simulants (95% aqueous ethanol). Values are presented as means with standard deviation.

Table 4.1.
Physical properties of non-modified chitosan films and pre-grafted GA-chitosan films and surface-grafted chitosan films.

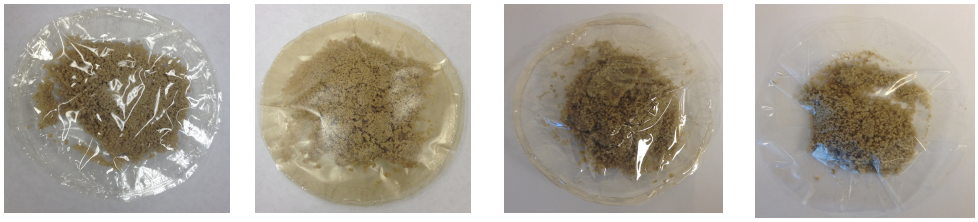
Different letters in columns for each type of the film denote significant difference ($p < 0.05$).

Films	Color			Thickness (μm)	Tensile strength (kg/cm^2)	Elongation %	WVP (Corrected, g mm/m^1d kPa)
	L	a	b				
Non-modified CF	89.86±0.80a	-1.78±0.29a	2.15± 0.23b	26.8±1.56b	1300±301a	3.19±1.35a	5.26±0.08a
Pre-grafted GA CF	83.40±1.31b	-1.31±0.22a	18.40± 2.26a	33.33±2.12a	566±148b	1.13±0.60ab	4.72±0.39a
Surface-grafted CF	87.59±0.97ab	-1.43± 0.04a	3.90±0.34b	26.6±1.4b	351±288b	0.71±0.39b	6.05±2.38a

Non-modified CF Pre-grafted CF Surface-grafted CF

Figure 4.4 Appearance of Non-modified chitosan films, pre-grafted chitosan films and surface-grafted chitosan films.



Pure chitosan films | Pre-grafted chitosan films | Surface-grafted chitosan films | Polyethylene films

Figure 4.5 Ground sunflower seeds packaged in non-modified chitosan, pre-grafted chitosan, surface-grafted chitosan and polyethylene pouches.

Table 4.2
Parameters of lipid oxidation in ground sunflower seeds during 20-week incubation at 50 °C and 50% RH.

Chitosan films	Time (weeks)	Conjugated dienes (CD extinction value)	Conjugated trienes (CT extinction value)	Peroxide value (PV meq/kg oil)	TBARS_Concentration(mg MDA eq/kg sample)
Non-modified	0	3.325 ± 0.11 d, A	0.204 ± 0.01 c, A	4.780 ± 0.35 bc, A	0.502 ± 0.03 b, A
	4	9.094 ± 0.62 c, B	0.565 ± 0.09 c, B	8.722 ± 0.26 a, B	0.743 ± 0.24 ab, A
	12	19.213 ± 1.63 b, BC	1.133 ± 0.31 b, C	5.201 ± 0.51 b, B	0.876 ± 0.05 a, A
	20	23.590 ± 0.47 a, A	1.884 ± 0.04 a, B	3.732 ± 0.78 c, B	0.492 ± 0.04 b, B
pre-grafted	0	3.325 ± 0.11 c, A	0.204 ± 0.01 b, A	4.780 ± 0.35 c, A	0.502 ± 0.03 b, A
	4	9.145 ± 1.76 b, B	0.550 ± 0.10 b, B	11.105 ± 1.48 a, AB	0.638 ± 0.04 a, A
	12	19.906 ± 0.46 a, AB	1.082 ± 0.14 b, C	7.057 ± 0.09 b, AB	0.512 ± 0.08 ab, B
	20	22.432 ± 1.19 a, A	3.145 ± 0.83 a, B	3.555 ± 0.53 c, B	0.429 ± 0.02 b, B
Surface-grafted	0	3.325 ± 0.11 c, A	0.204 ± 0.01 c, A	4.78 ± 0.35 c, A	0.502 ± 0.03 a, A
	4	9.316 ± 0.45 b, B	0.522 ± 0.06 c, B	13.300 ± 0.49 a, A	0.640 ± 0.09 a, A
	12	19.423 ± 1.82 a, AB	0.978 ± 0.10 b, C	7.333 ± 0.31 b, A	0.487 ± 0.05 a, B
	20	20.896 ± 2.22 a, AB	1.820 ± 0.28 a, B	4.074 ± 0.98 c, B	0.571 ± 0.10 a, B
PE	0	3.325 ± 0.11 c, A	0.204 ± 0.01 d, A	4.78 ± 0.35 c, A	0.502 ± 0.03 b, A
	4	8.230 ± 0.08 b, B	0.550 ± 0.10 c, B	11.671 ± 0.43 a, A	0.796 ± 0.09 a, A
	12	15.933 ± 1.13 a, C	1.082 ± 0.14 b, B	8.870 ± 1.38 b, A	0.433 ± 0.07 b, B
	20	15.360 ± 0.43 a, B	3.434 ± 0.01 a, B	5.043 ± 0.03 c, B	0.573 ± 0.07 b, B
Open	0	3.325 ± 0.11 c, A	0.204 ± 0.01 d, A	4.78 ± 0.35 c, A	0.502 ± 0.03 c, A
	4	12.722 ± 0.50 b, A	0.877 ± 0.07 c, A	8.658 ± 1.22 b, B	0.640 ± 0.015 bc, A
	12	22.714 ± 0.33 a, A	3.392 ± 0.13 b, A	8.418 ± 0.79 b, A	0.759 ± 0.09 b, A
	20	25.040 ± 3.02 a, A	10.270 ± 1.20 a, A	32.709 ± 1.18 a, A	2.160 ± 0.10 a, A

Different lowercase letters in columns denote significant difference in values during storage for each type of the film ($p < 0.05$). Different upercase letters within each column denote significant deference between samples packaged in different materials ($p < 0.05$).

CHAPTER V

OVERALL CONCLUSIONS AND RECOMMENDATIONS

Pure DI water as a grafting solvent was found to result in the highest grafting yield of 285.9 mg GA eq/g chitosan, since EtOH reduces the grafting efficiency of the reaction by acting as a reactant to produce ethyl gallate and decreasing the yield of GA-NHS ester. But a concentration of 25% EtOH in aqueous systems seems the most practical due to the high grafting efficiency (260.9 mg GA eq/g) and easily separable grafted chitosan. Using quantitative ^{1}H NMR, reaction monitoring and LR-HSQMBC by NMR, we were able to show the formation of GA-NHS ester with the highest yield of 32.7% at 1 h in D_2O and provide the foundation for improving the efficiency of EDC/NHS activation of GA by NMR studies. Additionally, surface grafting of GA onto chitosan films via EDC/NHS allowed us to produce transparent films with no significant color change, which avoided the decreased solubility of chitosan powder caused by EDC. By food storage test, we were able to prove the effect of surface-GA-grafted chitosan films on protecting sunflower seeds from lipid oxidation.

For future research, the first recommendation is to improve the efficiency of EDC/NHS activation of GA by changing pH or reagents ratio by NMR. The second recommendation is surface grafting GA onto films blended by chitosan and polyethylene for practical industrial application. Overall, the active packaging prepared by GA grafted chitosan films with GA via EDC/NHS may be a potential packaging material to maximize the prevention of lipid oxidation in different foods.

VITA

Ping Guo was born in Baoding, Hebei, China on February 1st, 1990. She graduated from Baoding No.1 High School. She continued her education at Nanjing Agricultural University where she earned a B.E. degree in Food Quality and Safety. She later earned an M.S. degree in Food Science and Technology with a concentration in Food Chemistry from the University of Tennessee, Knoxville. During her two-year graduate school, she found her interest in learning more about NMR programs and its application in natural products. Ping will pursue a doctoral degree in the application of NMR in Food Science.