

Water Soluble Chitosan Derivatives via the Freeze Concentration Technique

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Abstract—Chitosan has been an attractive biopolymer for decades, but its processability is lowered by its poor solubility, especially in physiological pH values. Freeze concentrated reactions of chitosan with several organic acids including acrylic, citraconic, itaconic, and maleic acid revealed improved solubility and morphological properties. Solubility traits were assessed with a modified ninhydrin test. Chitosan derivatives were characterized by ATR-FTIR and morphological characteristics were determined by SEM. This study is a unique approach to chemically modify chitosan to enhance water solubility.

Keywords—Chitosan, Freeze Concentration, Frozen Reactions, Ninhydrin Test, Water Soluble Chitosan.

I. INTRODUCTION

CHITOSAN, N-deacetylated form of its precursor chitin [1], is a widely used biological material with its remarkable properties of biocompatibility, biodegradability, low toxicity and many others [2]. Therefore, researchers have particularly focused on its biomedical applications, mostly in the areas of drug delivery systems, biotechnology, tissue engineering and wound dressing materials [3], [4].

Although chitosan is a promising candidate for several biomedical purposes with its promising properties, its processability is limited by poor water solubility. Crystallinity of chitosan that is established by high regularity of intermolecular Hydrogen bonds between the polymer chains is the main obstacle against solubility of biopolymer. Chemical structure of chitosan composes of two randomly distributed monomeric units; glucosamine and N-acetyl glucosamine, respectively [2]. The primary amino groups and free hydroxyl groups attached to glucosamine unit give the structure a wide H-bonding capacity; hence, they enhance the crystallinity of the structure. In addition, the nature of amino groups ($Pka=6.5$) gives this biopolymer a basic character [5]. Therefore, the crsytallinity of chitosan molecule has to be deranged by breaking the regularity of Hydrogen bonds within polymer chains to obtain water-soluble chitosan derivatives.

One of the main strategies to increase the solubility of chitosan is to modify the amino groups chemically. Adding bulkier side groups upon modification extends intermolecular spacing within chains, creating more water accessible area.

Although there are numerous synthetic methods to decrease H-bonding capacity of the biopolymer, one of the most direct approaches is to modify primary amino groups of the N-deacetylated monomeric units. In addition, this modification also allows the polymer to be soluble in a broader pH range by reducing the number of primary amine groups that are only soluble at acidic pH values.

The first step in a freezing of an aqueous solution is the crystallization of water molecules during which solvent molecules nucleate first, and followed by removal of ice crystals from the solution. Thus, the concentration of solute molecules increase significantly and such increase takes place until the temperature reaches eutectic point. This effect is called as freeze concentration (FC) [6] and has found its applications for decades in water separation applications [7] and in food industry [8]. FC solutions are powerful tools for chemical reactions since very high concentrations and higher viscosities of reagents allow chemical reactions take place faster in spite of the low temperature. That is to say, the increase in both concentration and viscosity drives FC systems to thermodynamically more favorable states and allow chemical reactions to take place at subzero degrees.

Frozen solutions take the advantage of enhanced concentration in spite of their low temperature. A proof of this increase in concentration in the literature was the significant difference between rates of frozen solution and super cooled liquid reactions of ethylene chlorohydrin with sodium hydroxide [9]. Also, it was recently verified that freeze concentration effect enhanced the formation of IBr_2^- and this finding explained the chemistry behind ozone depletion events in polar troposphere [10]. Along with these, several FC reactions have been carried out and explained in the literature, especially focusing on cryogel synthesis applications [11], [12]. The purpose of this paper is to demonstrate the validity of FC method on organic modifications of biomacromolecules. For this purpose, Michael addition of chitosan with acrylic acid proposed by [13] was selected as the model system, modified to low temperatures and expanded with several organic acids. Results showed that FC reactions were performed successfully, and the products obtained by FC effect showed better solubilities in physiological pH values and several morphological differences were observed than those obtained by conventional method.

II. MATERIALS & METHODS

A. Chemicals

Chitosan and all the small organic molecules were purchased from Sigma & Aldrich, Germany. Chitosan, from

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crab shells, was in practical grade with degree of deacetylation ≥ 85 and viscosity > 200 . All the solvents, organic reagents and crosslinking agents were in practical grade.

TABLE I
SAMPLE NAMES, AMOUNTS OF REACTANTS USED, AND REACTION
CONDITIONS FOR CHITOSAN MODIFICATION REACTIONS

Sample Name	Reactant (10%, 1:1)	Reaction Temperature ($^{\circ}\text{C}$)
A-5	Acrylic acid	-5
A50	Acrylic acid	50
C-5	Citraconic acid	-5
C50	Citraconic acid	50
M-5	Maleic acid	-5
M50	Maleic acid	50
I-5	Itaconic acid	-5
I50	Itaconic acid	50

B. Preparation of N-carboxy Derivatized Chitosan

Michael addition was carried out according to [13]. Control samples were obtained and compared to those prepared with the same procedure, except the temperature was kept at, -5°C . In brief, 2 g of chitosan was dissolved in 100 mL of 10% acetic acid solution. After the filtration, a solution of each reactant (10% wt.) was added into chitosan solutions. The resulting solutions were divided into two parts. First part of each chitosan reaction mixtures were stirred at 50°C overnight, whereas second parts were stirred at -5°C . Details of reaction conditions and mixtures are found in Table I. After the reactions were completed, 0.5 M NaOH were added into mixtures to adjust the pH at 6.0. The mixtures were then dialyzed using a dialysis membrane (MW cutoff= 3.5K) 2 times against 0.1 M NaCl and 3 times against $\text{d-H}_2\text{O}$, respectively. Products were freeze dried to obtain dry N-carboxy derivatized chitosan powders/ lyophilizates.

C. Cadmium-Ninhydrin Test and Color Quantification

Cd-ninhydrin test [14] was used to compare the solubilities of Chitosan derivatives with native chitosan. This test is a direct method to detect the amine residues resulting with a red and pale orange/yellow color for primary and secondary amines, respectively. 1 mg/mL solutions of chitosan samples with pH 7.4 were dropped on a Whatman 3MM paper, freshly prepared Cd-ninhydrin reagent was sprayed on sample spots and dried. Cd-ninhydrin test yielded a range of colors from red to yellow allowing us to quantify the amount of primary and secondary amines depending on the red color intensities.

A color intensity analysis method was adopted similar to Western blot quantification described by [15] to quantify ninhydrin yields. Freshly prepared Cd-ninhydrin sprayed Whatman papers prepared as described above were scanned and processed with ImageJ software [16]. Mean color intensities were measured for Cd-ninhydrin added bands of native and FC derivatized chitosans.

is related to asymmetric C-O vibrations resulting from deacetylation and the double peak around $1069\text{--}1028\text{ cm}^{-1}$ is attributed to C-OH and -C-O-C vibrations of the β -(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) rings. The absorption bands at 1381 and 1422 cm^{-1} are attributed to $-\text{CH}_2$ and $-\text{CH}$ bending vibrations, respectively. The overlapped peaks with frequencies of $1589\text{--}1650\text{ cm}^{-1}$ correspond to the primary amine groups and carbonyl absorption of N-acetamido groups, respectively. The shouldered peak at $2872\text{--}2920\text{ cm}^{-1}$ corresponds to symmetric and asymmetric stretchings of the aliphatic $-\text{CH}_2$ and $-\text{CH}_3$ groups. The broad peak around 3362 cm^{-1} is originated from $-\text{OH}$ stretchings of free carboxyls bonded by hydrogen bonding.

Different than native chitosan, FC derivatized chitosan spectra presented additional vibrational bands. The primary amine signal at 1589 cm^{-1} in native chitosan disappeared or decreased in intensity, whereas a band corresponding to N-H bending vibrations in secondary amines appeared around 1550 cm^{-1} following FC modification. In addition, the increase in the intensity of amide carbonyl stretch around 1650 cm^{-1} in FC derivatized chitosan samples also supported the successful addition of FC products. Moreover, the newly formed bands around 1310 cm^{-1} which arose from the introduction of secondary amines in the FC derivatized chitosan products made a significant contribution to successful derivatization. Brief summaries of main spectral data proving the FC derivatization are listed in Table II.

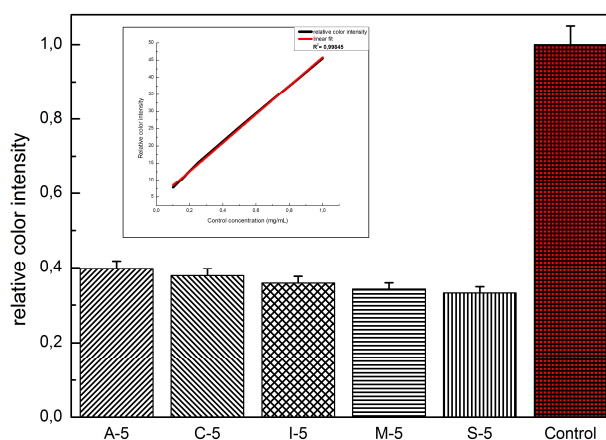


Fig. 1 Relative color intensities of 1 mg/mL of native chitosan (control) and FC derivatized products, calibration curve assessing validity of color intensity quantification method (inset)

III. RESULTS & DISCUSSION

A. Characterizations

Fig. 2 (a) shows the attenuated total reflectance (ATR) FTIR spectrum of native chitosan. The band around 1151 cm^{-1}

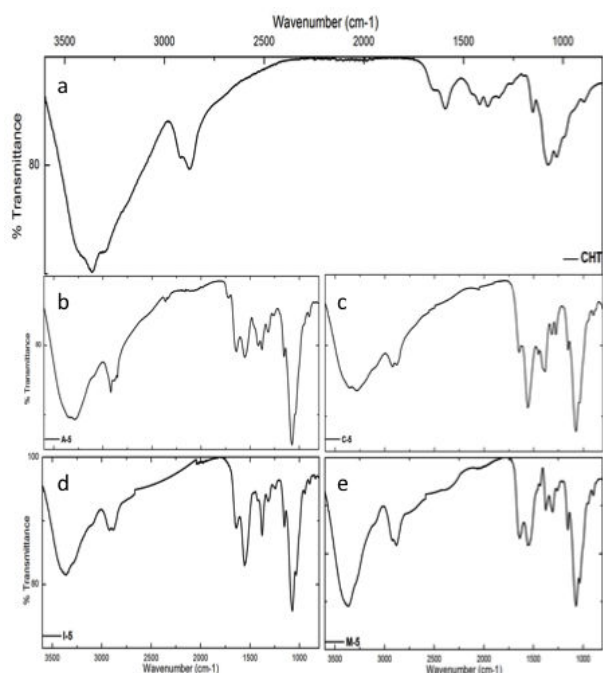


Fig. 2 FTIR spectra of native chitosan (a), and Michael adducts of (b) acrylic acid, (c) citraconic acid, (d) itaconic acid, (e) maleic acid

B. Solubility Traits

The color intensities of Cd-ninhydrin added FC derivatized sample bands were measured by ImageJ tool, and their mean color intensities were recorded and compared to native chitosan of the same amount. Details were shown in Fig. 1. Results suggested that 1mg/mL samples of FC derivatized products were in significantly lower color intensities than that of same amount of chitosan. This can be explained by less amount of ninhydrin yield of FC derivatized samples, which is directly proportional to a less amount of primary amine groups in the structures than that of native chitosan. The result also confirmed the success of FC reactions and served up as a contributive evidence to increased solubility of FC derivatized chitosan products.

To assess the validity of color quantification method, a standard curve was constructed using native chitosan samples. For this purpose, 30 μ L of native chitosan samples in different concentrations were dropped on whatmann a paper and same procedure was applied as described in methods section. As seen in Fig. 1. (inset), Color quantification method resulted with a linear fit, with $R^2 = 0.99845$, suggesting that the method responds linearly to constant increase of amine concentration and allowed us to interpret the amount of primary amines in FC derivatized products.

C. Morphology

Morphological properties of native, high temperature and FC derivatized chitosans were studied by scanning electron microscopy (SEM). Images of native chitosan and FC derivatized samples were shown in Fig. 3 and 4, respectively. SEM micrographs of native chitosan revealed smooth surfaces

with no fibrillar structure whereas derivatized chitosans showed microphorous fibrillar structure clustered to compose porous structure. Compared to chitosan derivative obtained at 50°C, FC derivatized carboxyethyl chitosan displayed a more regular pore structure. In addition, fibril sizes were relatively smaller in FC derivatized chitosan. Results indicated that morphology of chitosan was altered upon chemical modification and the differences in fibrillar structure and pore regularity may be explained by the effect of ice formation during FC derivatization leading to more regular and pore size and fibrillar structure.

TABLE II
IMPORTANT IR SPECTRAL DATA OF NATIVE AND FC DERIVATIZED CHITOSAN

Code	IR ABSORPTION				
	-CO	-NHR	-NH-	-NH ₂ -	NH-CO
CHT	1069	-	-	1589	1650
A-5	1072	1311	1550	-	1644
C-5	1072	1314	1551	-	1644
M-5	1069	1305	1553	-	1638
I-5	1072	1314	1553	-	1638

IV. CONCLUSIONS

Solubility of chitosan is an important obstacle against its utilization especially as a biomaterial and therefore limits its applications despite its intriguing properties. Ninhydrin results showed that solubility of chitosan was significantly increased by Michael addition of several organic acids. According to this test, since the decrease in the number amount primary amine sites serves to lower crystallinity, solubility of chitosan samples can be related with decreased ninhydrin yields. In addition, it was also confirmed by FTIR that chitosan modification successfully took place in FC conditions. Soluble chitosan derivatives have been synthesized through different reaction methods, but to our knowledge, this study is unique in terms of employing freeze concentration technique as a reaction tool to macromolecules.

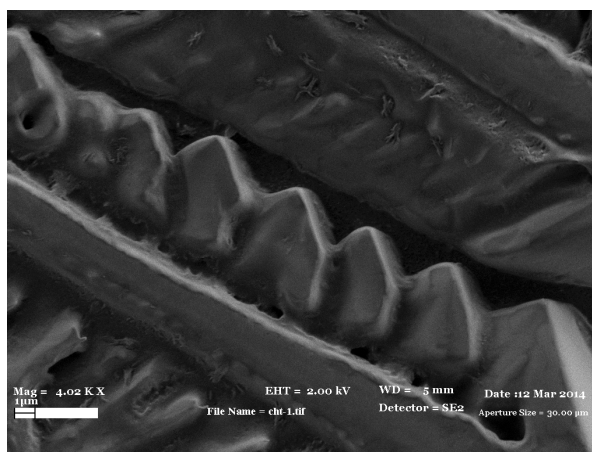


Fig. 3 Scanning electron microscope (SEM) image of native Chitosan

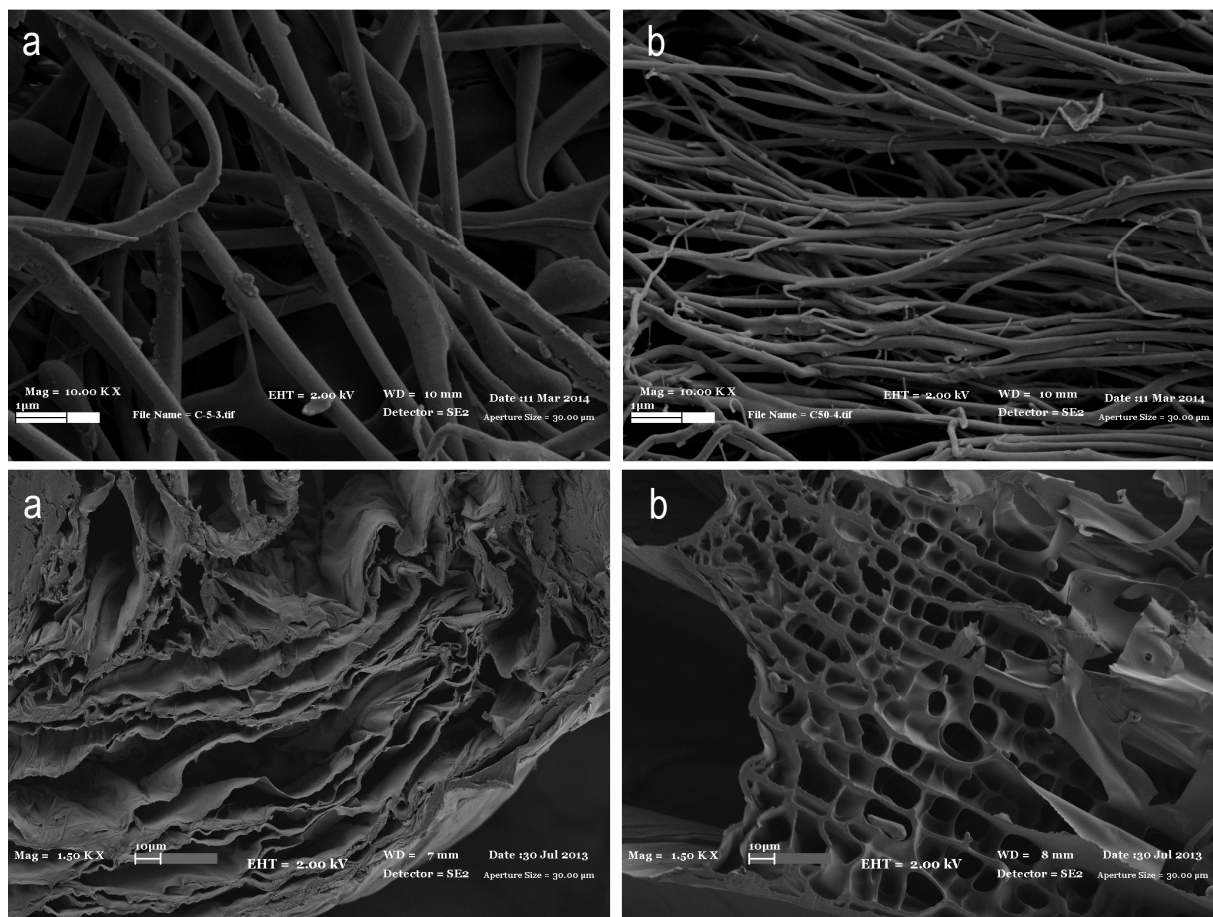


Fig. 4 SEM micrographs of (a) 50°C derivatized and (b) FC derivatized products at several magnifications

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