

Synthesis of a Novel Amphiphilic Nano-Chitosan Material

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Abstract. Chitosan (CS) is a semi-synthetic polysaccharide derived from the most abundant naturally occurring polymer, chitin. It has found applications in fields as diverse as health, cosmetics, agriculture, environmental remediation etc. While biodegradability is important for non-toxic polymer-based material, stable and mechanically resilient chitosan is needed for harsh environmental applications. We synthesized a nano-chitosan derivative (LA-CS-Suc) with comparable thermal stability as the parent polymer. Synthesis involved chemically linking an organic acid to CS via carbodiimide chemistry. LA-CS-Suc was characterized by FT-IR, TGA, DSC and DLS. Cellular toxicity was also carried out. Successful conjugation was confirmed by FT-IR spectroscopy. Thermal stability was comparable to parent polymer. LA-CS-Suc had particle sizes ranging from 215 to 320 nm and they showed no cellular toxicity.

1. Introduction

Chitin is the major macromolecule in the exoskeleton of crustaceans, insects, and fungi. CS is obtained by deacetylation of chitin. Greater than 50% deacetylated chitin is classified as CS. CS has wide applications because it is biodegradable, biocompatible and non-toxic.[1] These properties along with its amenability to chemical modifications make it an ideal starting polymer for the development of environmentally friendly materials.[2]

Many chemical modification strategies of CS have involved substitution on the C-2 primary amine—a readily reactive nucleophile. Less reactive, but also available for chemical derivatization, are the C-3 and C-6 hydroxyl groups.

Zhong et al explored the electrostatic potential of maleic-CS nano-fibres in measuring proton current flow.[3] They demonstrated a solid-state chitosan-based ionic flow modulator that shows potential in biomedical applications. CS-based material have also shown potential in fuel cell applications as a stable, mechanically resilient and environmentally safe component for the modulation of cationic and anionic conductivities.[4, 5]

Here, we report the chemical synthesis and characterization of polycationic and polyanionic nano-CS which retain the mechanical properties of the parent CS polymer.

2. Materials and Methods

Chitosan of molecular weight 10 kDa and >90%deacetylation was obtained from D. B. Fine Chemicals, South Africa. All other chemicals were purchased from Sigma-Aldrich. Ethanol (EtOH, 99.5%), and acetone (99.5%) were purchased from Merck. Distilled and deionized water (dH₂O) was used in experiments. Ultraviolet spectrum was measured by using the Mettler Toledo UltraViolet5Bio (UV5Bio). Malvern Zetasizer Nano ZS was used to determine the hydrodynamic particle size of the



material. Infrared absorption spectra were measured using the Perkin Elmer Spectrum 100 Fourier Transport Infrared (FTIR) Spectrometer. The Mettler Toledo thermogravimetric analysis (TGA) SDTA851^e was used to obtain the degradation thermogram of the material. Differential scanning calorimetry (DSC) Q2000 TA Instrument was used to determine the melting point of the material.

2.1 Synthesis of Lauryl-CS:

CS (2.0 g, 11.2 mmol, 1.0 eqv) was completely dissolved in aqueous acetic acid solution (1%, v/v, 100 ml) and lyophilized. The dried chitosan was then dissolved in dH₂O (200 ml) and DMF (40 ml) was added to the solution slowly without precipitating the polymer. To a stirring solution of LA (120 mg, 0.56 mmol, 0.05 eqv) in DMF (10 ml) was added EDC·HCl (233 mg, 1.4 mmol, 0.15 eqv) and NHS (207 mg, 1.8 mmol, 0.15 eqv) and the reaction was left to run for 2 hours. The EDC-activated LA solution was added dropwise to the stirring solution of lyophilized chitosan and the reaction was left to run for 24 hours. The LA-conjugated CS was precipitated with cold EtOH and centrifuged at 6000 rpm for 20 min, twice. After the final wash residual solvent was removed *in vacuo* to obtain the pure lauryl-CS (LA-CS).

2.2 Synthesis of Succinylated LA-CS:

LA-CS was suspended in a solution of dH₂O (100 ml), DMF (40 ml), made alkaline using TEA. Succinic anhydride (223 mg, 2.24 mmol, 0.20 eqv) was added to the stirring solution and the reaction was left for 24 hours. LA-CS-hemisuccinic acid (LA-CS-Suc) was precipitated using cold EtOH and centrifuged at 6000 rpm for 20 min, twice. The residual solvent was removed *in vacuo* and the product was then placed in an oven at 40 °C to dry.

2.3 Thermal studies:

Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were conducted for all derivatized materials to assess decomposition rate, moisture content and melting point temperature. DSC comprised of a heating-cooling-heating cycle from -60 °C to 300 °C at a rate of 10 °C/min. Nitrogen gas flow was set to 25 ml/min. TGA of derivatized and non-derivatized CS were subjected to temperatures of ambient to 400 °C.

2.4 Particle size and surface charge:

Particle size and surface charge analyses were conducted by dissolving 1 mg each of CS, CS-LA, LA-CS-Suc in 1 ml of 1% acetic acid (for CS), 1 ml of 4% acetic acid (for CS-LA), 1 ml of PBS (pH = 7.4) and 1 ml of NaCl (1.0 M) (for LA-CS-Suc). Sodium bicarbonate was added to the CS-LA-Suc to enhance its aqueous solubility. All the solutions were stirred for 20 min at room temperature and analysed using refractive index of 1.330 and absorption value of 0.001.

2.5 Cell toxicity:

A haemolytic assay was used to determine the toxicity of LA-CS-Suc to red blood cells (RBCs). Non-derivatized CS was used as reference control. A 2% (v/v) RBC solution was prepared. LA-CS-Suc and CS were suspended in PBS at 10 mg/ml stock suspension (pH 7.4). The LA-CS-Suc and non-derivatized were serially diluted from the 10 mg/ml stock solution. A 1% (v/v) solution of Triton X-100 was used to obtain 100% haemolysis. 1200 µl of each sample was added to 300 µL of RBC solution. The suspensions were then incubated with shaking at 37 °C for 2 hours. Thereafter, all samples were centrifuged at 5000 rpm for 3 min at 20 °C. The supernatants (1 ml) were withdrawn and analysed on a UV spectrophotometre at 570 nm for haemoglobin content. The results obtained were expressed as percent haemolysis. Zero percent haemolysis was taken as the absorbance of the supernatant of the RBC incubated in PBS (pH = 7.4) solution, while 100% haemolysis was expressed as the absorbance of the supernatant of RBC treated with 1% solution of Triton X-100.

3. Results and Discussion

CS was successfully modified with LA using carbodiimide chemistry to yield the product LA-CS (Figure 1). LA-CS was further acetylated with the dicarboxylic acid succinic acid with the reagent succinic anhydride. The EDAC and anhydride were used because they are less harsh on the polymer than alternative coupling reagents like acid chlorides. Yields of up to 70% were obtained in these reactions.

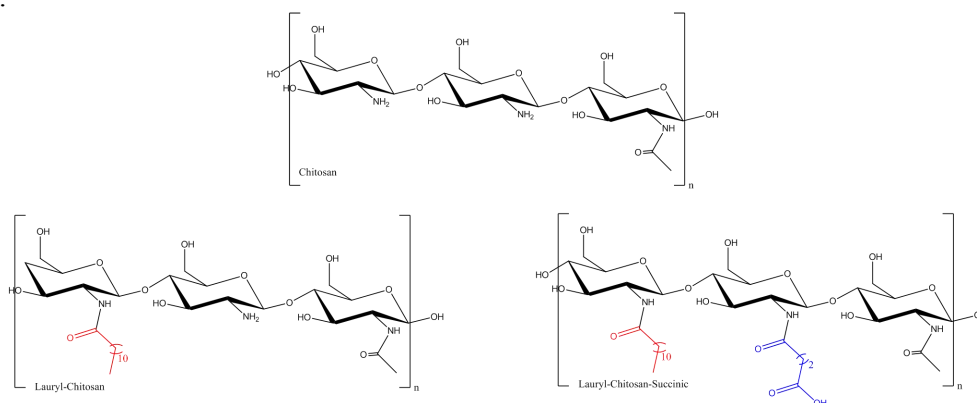


Figure 1. Chemical structures of chitosan and its chemical derivatives.

3.1 Characterization

Confirmation of successful product formation was obtained by FT-IR spectroscopy (Figure 2). Transmission peaks at 1640-1655 cm^{-1} for both modified chitosan correspond to the carbonyl ($\text{C}=\text{O}$) stretching as a result of amide bond. The peak around 3350 cm^{-1} corresponds to the stretching of the sugar monomers' hydroxyl groups ($\text{O}-\text{H}$). The occurrence of peaks 2929 cm^{-1} for LA-CS-Suc and 2978 cm^{-1} for CS-LA is a result of $\text{C}-\text{H}$ stretching for LA and succinic acid. The absence of peak 1770 cm^{-1} , a characteristic peak of an ester bond, supports N-acylation of CS with LA and succinic acid.

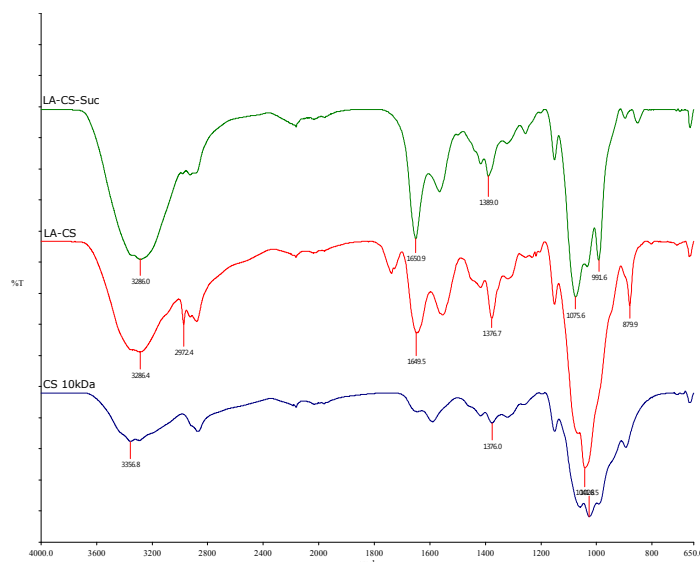


Figure 2. FT-IR spectrum of chemically modified chitosan with LA and succinic acid. CS-LA: CS derivative of LA. LA-CS- Suc: CS derivative of lauric and succinic acid

Thermal stability of non-derivatized and derivatized CS was confirmed by TGA and DSC (Figure 3 and 4). The initial reduction in weight of 2-9% for succinic acid, CS, LA-CS and LA-CS-Suc was proposedly due to moisture content. LA showed near 100% weight reduction within 50 $^{\circ}\text{C}$. Succinic

acid showed a greater weight loss between 200-230 °C. CS's greatest thermal degradation appeared between 270-320 °C with over 30%. Similar percent reduction in weight loss up to 70 °C was observed for LA-CS-Suc. CS (> 80 °C) and LA-CS-Suc (>325 °C) experienced a gradual reduction in weight loss. This weight loss patterns are primary due to degradation, depolymerisation and dehydration of acylated units of the polysaccharides. Although thermal stability for LA-CS-Suc seems to be significantly less compared to CS, the multiple degradation patterns of LA-CS-Suc proposes a change in transition state, as a function of temperature. This is supported by the DSC thermogram of LA-CS-Suc (Figure 4).

3.2 Thermal studies

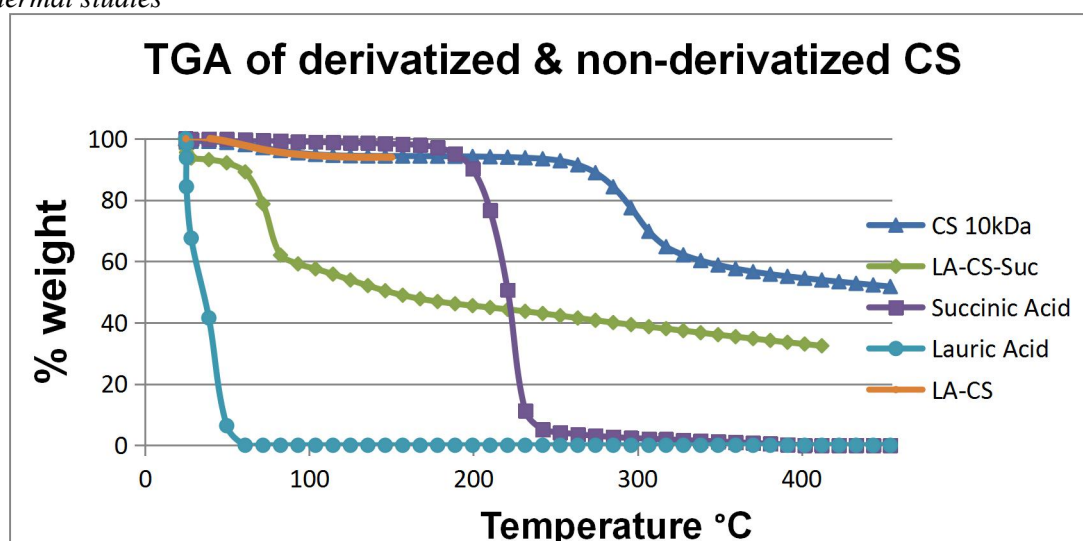


Figure 3. TGA of CS, LA-CS- Suc, lauric and succinic acid.

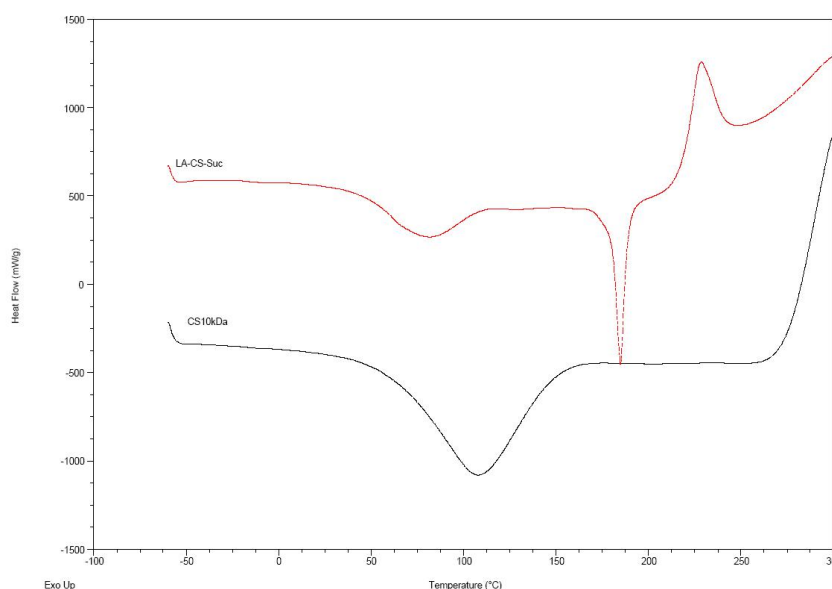


Figure 4. DSC thermogram of CS10kDa and LA-CS-Suc

DSC thermal decomposition of LA-CS-Suc shows two distinct peaks at approximately 175 °C and 230 °C. This thermogram suggests two ideas of thermal degradation of LA-CS-Suc. The peak around 175 °C proposes crystallinity of the modified CS. The exothermal peak around 230 °C suggests

decomposition of this crystalline conformation. These observations as a function of temperature, suggests LA-CS-Suc undergoes change in transition states from an ordered to an unordered conformation (crystalline to amorphous). This proposes energy absorption and release characteristics of LA-CS-Suc with the increase in temperature. Similar peaks are not observed for CS10kDa.

3.3 Particle size

Table 1. Particle size and surface charge of CS derivatives in various aqueous diluents.

Sample Type	Diluent	Average particle size (d.nm \pm SD)	Average Particle surface charge (\pm SD)
LA-CS-Suc	dH ₂ O	320.4 \pm 28.43	-20.77 \pm 0.40
LA-CS-Suc	PBS	216.9 \pm 17.49	-14.93 \pm 1.84
LA-CS-Suc	NaCl	257.6 \pm 5.71	-12.47 \pm 0.53
LA-CS	4% Acetic Acid	371.6 \pm 21.49	19.3 \pm 3.40
CS 10kDa	1% Acetic Acid	1078.67 \pm 5.44	26.93 \pm 1.20

Synthesis of nano-chitosan was confirmed by dynamic light scattering on a zetasizer (Table 1). LA-CS was determined to be 371 nm while the LA-CS-Suc was smaller at between 216 and 320 nm depending on the solution used for analysis. LA-CS-Suc was investigated in PBS, 1.0 M NaCl, and deionized water with particle size increasing over about 100 nm range. Non-derivatized CS was observed at over 1 μ m.

A significant property of the particles was stability in surface charges. Non-derivatized CS is a polyelectrolyte with net cationic charge in acidic solutions. Its pKa is 6.5. This explains the high positive charge observed. However, this charge is reduced when LA is conjugated to CS. This is in part due to some of the $-NH_2$ groups been converted to amides after conjugation to LA. There is a switch to strong anionic surface charge when succinic acid is introduced. The high charge values confirm the formation of stable nanoparticles.

CS in aqueous solutions has a uniform distribution of functional groups capable of forming hydrogen bonding with water. This thermodynamically stable linear conformation is disrupted by the introduction of the hydrophobic LA. LA constrains the polymeric backbone to adopt a particulate conformation that sequesters the aliphatic chain into the core, minimizing exposure to the aqueous environment (Figure 5). The charged amines of LA-CS and carboxylates of LA-CS-Suc result in repulsion between particles. This effectively prevents particle aggregation and enhances discrete nanoparticle formation.

The anionic charge and size of LA-CS-Suc was found to be very stable in solutions of different ionic salt strengths. In deionized water the charge and particle size were greatest. This could be attributable to a near total lack of counter ions which would stabilize individual particles. However, the presence of Na^+Cl^- ions in the 1 M NaCl solution provides small counter ions that separate and stabilize particles. Further, in the phosphate-buffered solution a much bigger PO_4^{3-} complex interferes with particle aggregation and reduces the particle size even more.

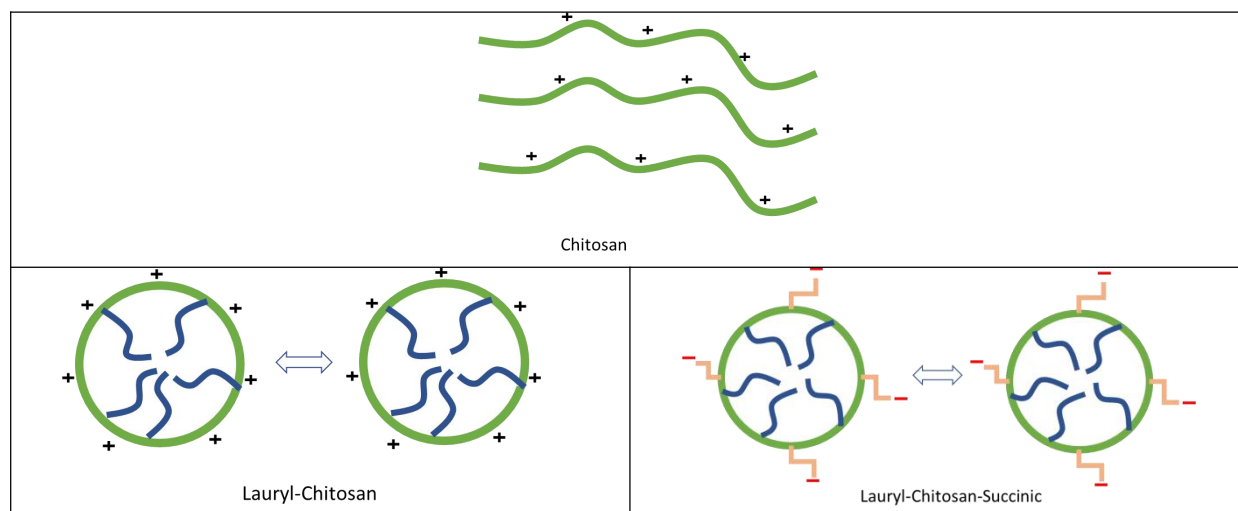


Figure 5. Schematic representation of the proposed conformations of chitosan (CS), Lauryl-chitosan (LA-CS), and Lauryl-Chitosan-Succinic acid (LA-CS-Suc).

3.4 Cell toxicity

The toxicity of the LA-CS-Suc derivatives was investigated with RBCs. Haemolysis is known to be related to high cationic charge.[6] Because LA-CS has a similar cationic charge to CS we only investigated LA-CS-Suc. There was no significant difference observed between the haemolysis of CS and LA-CS-Suc at concentrations up to 10 mg/ml (Figure 6). The extent of haemolysis obtained was less than 10% and similar to the degree of haemolysis seen with the PBS negative control. This clearly indicates that the synthetic derivatization of CS did not reduce its safety profile.

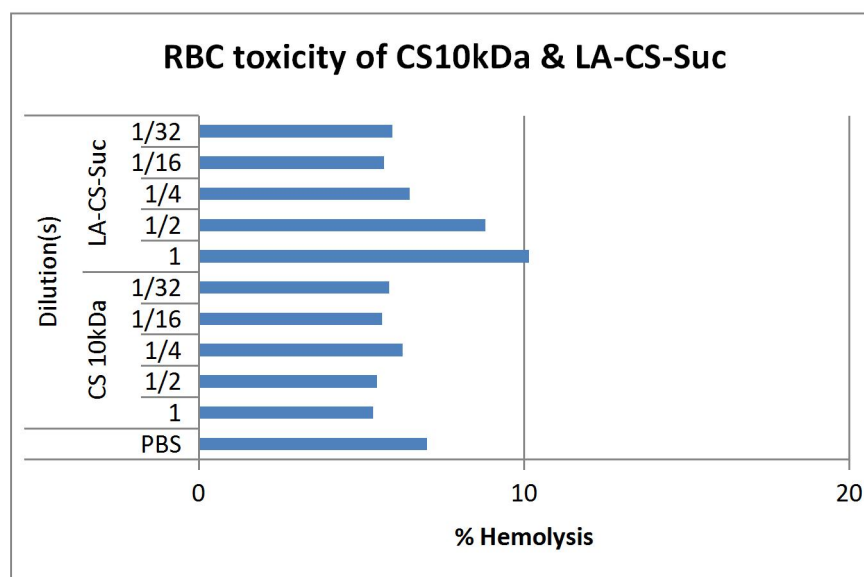


Figure 6. Haemolysis assay of CS10kDa and LA-CS-Suc.

4. Conclusion

In this work we successfully synthesized and characterized cationic and anionic nano-CS derivatives. These particles did not lose the attractive properties of the parent chitosan like thermal stability and low toxicity. It is envisaged that the increased charge to particle ratios of these particles will make them attractive as green, biodegradable electrolytes. This could potentially make them applicable in

electronics, and fuel cells. Also, they could find application in health systems where charged delivery systems are required for tissue-specific delivery of bioactives.

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