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Free radical-induced chitosan depolymerized products protect calf thymus DNA from oxidative damage

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Abstract—Low molecular weight chitosan (LMWC) and chitooligosaccharides (COs), obtained by persulfate-induced depolymerization of chitosan showed scavenging of $OH^{\cdot}$ and $O_2^{\cdot -}$ radicals and offered protection against calf thymus DNA damage. Over 85% inhibition of free radicals and DNA protection were observed. LMWC (0.05 μmol) showed a strong inhibitory activity compared to COs (3.6 μmol). Further, LMWC showed calf thymus DNA condensation reversibly giving stability, as evident from CD, TEM and melting curves (T_m). A fluorescence study suggests the binding of LMWC in the minor groove, forming H-bonds to the backbone phosphates without distorting the double helix structure.
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1. Introduction

The enormous abundance of chitin and its unusual chemical properties, which can readily be modified, are factors underlying the current interest in extending its commercial potential. It is recognized that chitin is a linear copolymer composed of approximately 70–90% *N*-acetyl-D-glucosamine (GlcNAc) and 10–30% D-glucosamine (GlcN) units connected through (1→4)-β-glycosidic linkages, whereas its derivative chitosan is a de-N-acetylated product derived by hot alkali treatment. Most commercial grades of chitosan contain 75–95% of GlcN and 5–25% GlcNAc units. Partially depolymerized chitosans or low molecular weight chitosans (LMWC), with an average molecular weight of 10 kDa and chitooligosaccharides (COs) seem to have an enhanced biochemical significance compared to native chitosan.[1] LMWC and COs were proposed for practical use in milk preservation, cotton textile finishing and oral hygiene.[2–5] Due to their lower solution viscosity, low molecular weights, short chain lengths and easy solubility in neutral aqueous solution, they seem to be readily absorbed in vivo. Partially depolymerized chitosans modulated plant resistance to disease,[6] when administered to animals, they stimulate murine peritoneal macrophages leading to the killing of tumour cells[7] and showed an antitumour activity.[8–11] Chitin and chitosan oligomers possess additional functional properties such as immuno enhancing effects, and enhancing protection against infection with some pathogens in mice,[12,13] antifungal[14,15] and antimicrobial activities.[16]

1.1. Antioxidant activity

The physiological burden created by free radicals causes an imbalance in homeostatic phenomenon between oxidant and antioxidant species in the body. This imbalance leads to oxidative stress that is being suggested as the root cause of aging and various other human disorders like atherosclerosis, stroke, diabetes, cancer and neurodegenerative disease such as Alzheimer's disease

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and Parkinsonism.[17] It is known that the antioxidant effect of chitosan varies with its MW and viscosity, as shown in cooked comminuted fish flesh model system,[18] which is attributed to differences in the availability of net cationic amino groups, which impart intermolecular electrostatic repulsive forces leading to an increase in the hydrodynamic volume of the extended chain conformation. Perhaps this phenomenon may be responsible for lesser chelation by native chitosan, in addition to its high viscosity and high MW. Furda[19] has reported the degree of polymerization of glucosamine units as the major factor determining the viscosity of chitosan, whereas the degree of deacetylation is yet another factor that may be involved in the chelating ability of chitosan. Moreover, Xue et al.[20] have reported that water soluble chitosan may chelate metals or combine with lipids to display a significant antioxidant effect. $K_2S_2O_8$ was shown to induce chitosan depolymerization giving rise to LMWC (MW 40,000 Da, DA $\sim$1%) and COs mixture with degrees of oligomerization, as revealed by HPLC and MALDI-TOF-MS, 5–6 and higher oligomers, with an average MW of 1500 Da.[21,22]

Physiological defence systems with antioxidants composed of enzymes and vitamins will act against oxidative stress, namely, (1) suppressing generation of reactive oxygen species (ROS), (2) scavenging and clearance of ROS, (3) repairing and reconstitution of damaged products and (4) induction of antioxidant proteins and enzymes. However, the quantity of the latter under normal physiological conditions is sufficient only to cope with the normal threshold of physiologically generated free radicals. Therefore, any additional burden of free radicals, either from an indigenous or exogenous source can aggravate the situation, ultimately leading to cellular damage and subsequently cell death (prooxidant and anti-free radical (antioxidant) balance leading to oxidative stress).

Synthetic antioxidants generally used are pure, cheap and readily available, although natural antioxidants are considered much safer and most acceptable. Recently the antioxidant property of chitosan and its derivatives has attracted increasing attention,[18,23,24] wherein the scavenging mechanism is related to the fact that the free radicals can react with the NH_2 groups in chitosan to form a stable macroradical. This H-abstraction effectively intercepts a free radical before it can react with the substrate. The scavenging effect is better expressed by LMWC and COs, due to their ready and easy solubility in water, in comparison with native chitosan, which has a very high molecular weight and forms solutions that are highly viscous.

1.2. DNA condensation activity

DNA condensation provides a promising means whereby DNA containing genes of therapeutic interest can be prepared for transfer from solution to target cells for gene therapy applications. The phenomenon of condensation is difficult to distinguish from aggregation or precipitation. Condensation agents generally work either by decreasing repulsions between DNA segments (e.g., neutralizing of phosphate charge, and/or reorienting water dipoles near DNA surfaces by multivalent cations) or by making DNA–solvent interactions less favourable (e.g., by adding ethanol, which is a poorer solvent than water for DNA, or by adding another polymer, such as polyethylene glycol (PEG), which excludes spatial volume to the DNA).[25] Multivalent cations may also facilitate condensations. In aqueous solutions, condensation normally requires cations of charge 3+ or greater. Those most commonly used in condensation studies are the naturally occurring polymers spermidine^{3+} and spermine^{4+}. Divalent metal cations do not provoke condensation in water at room temperature except under special circumstances (in water–alcohol mixtures).[26] Usually DNA condensation in the presence of multivalent cation (spermine^{4+}, spermidine^{3+}) is determined by the total charge neutralization of the DNA, rather than by the binding of the multivalent cation per se.[26]

Chitosan-based transfection systems[27] provide certain advantages such as non-immunogenicity, lack of biohazards and the possibility for introducing larger DNA fragments into targets, over viral vectors in gene therapy. Although electrostatic association between cationic chitosan and negatively charged phosphates of DNA provides the primary driving force for complex formation, other interactions also seem to contribute. Binding of chitosan to DNA leads to condensation and the formation of compact, dense structure as revealed by electron microscopy.[27] Previously it was shown that chitosans of high molecular weights as well as specific salt forms are toxic.[28] The observation that highly purified LMWC used are neither toxic nor haemolytic and that they have the ability to complex DNA and protect against nuclease degradation further validates LMWC as components of a synthetic gene delivery system.[27]

The principle objective of this study was to establish a biopharmacological utility of LMWC and COs, derived from free radical-induced depolymerization of chitosan.

2. Results and discussion

2.1. Antioxidant activity

In the present study, both LMWC and COs were shown to have scavenging activity in the concentration range between 0.4–2.0 and 1–8 mg, respectively, which increased to over 80% of free radical inhibition (Figs. 1 and 2) with reference to curcumin (Table 1), a known antioxidant. The concentrations of 1.8 mg LMWC and 6 mg COs gave maximum inhibition of 86% each,

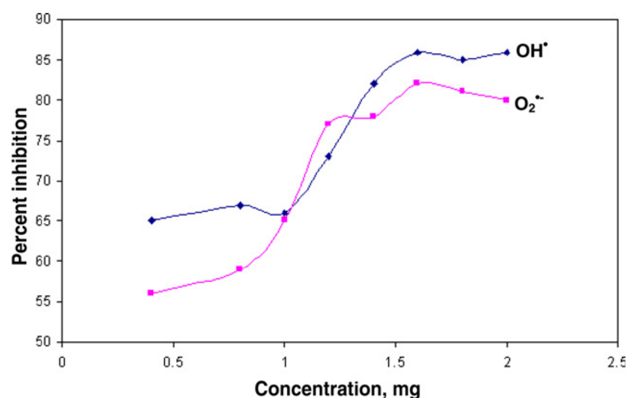

Figure 1. Scavenging effect of LMWC on hydroxyl and superoxide radicals. Values are means ± SD of three determinations.

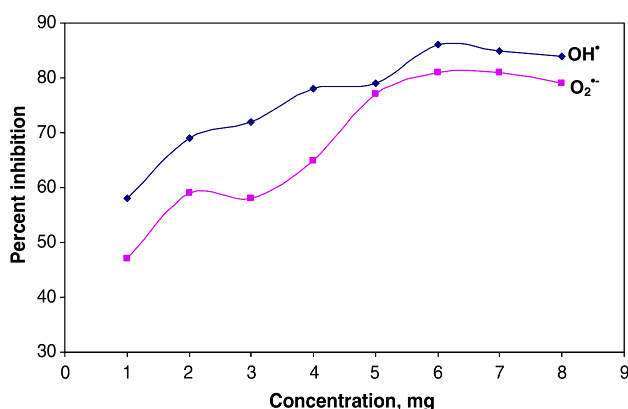

Figure 2. Scavenging effect of COs on hydroxyl and superoxide radicals. Values are means ± SD of three determinations.

Table 1. Antioxidant activity of LMWC and COs

Antioxidants	Concentration, mg (μmol)	Percent inhibition	
		$OH^{\cdot}$	$O_2^{\cdot -}$
Curcumin	0.1 (0.27)	95	92
LMWC	1.8 (0.05)	86	82
COs	6.0 (3.60)	86	81

respectively. Further increase in concentration did not result in any enhanced activity. Comparatively, LMWC was much more antioxidant than COs, probably for the reason that the available cationic groups in the former was several folds more and may be optimum.[9] The reduction of nitroblue tetrazolium by superoxide anion was used as a control reaction. However, the rate of scavenging of superoxide by ascorbate was much smaller than that by superoxide dismutase by a factor of about 10^4.[29] It is known that chitosan significantly decreases liver TBA reactive substances and increases antioxidant enzyme activities (catalase and superoxide dismutase).[23] LMWC/COs at similar concentrations (0.4–2.0 mg/1–8 mg, respectively) were also effective and showed increased trend up to over 80% inhibition of superoxide radicals (Figs. 1 and 2, Table 1).

Oxidative DNA damage is an inevitable consequence of cellular metabolism with a propensity for increased levels of endogenous ROS, wherein $OH^{\cdot}$ reacts by adding to double bonds of DNA bases by abstraction of a H-atom from the methyl group of thymine and C–H bonds of 2′-deoxyribose and also consequent modification of DNA bases leading to DNA damage.[29] Any such radical, that escapes natural scavenging by ascorbic acid can react with CTDNA, which gets oxidized and damaged and therefore moves faster (Lane B) on the agarose gel (Fig. 3). LMWC and COs added to the reaction mixture were capable of protecting CTDNA from such oxidative damage, in a dose dependent manner, with three concentrations each of LMWC and COs (Lane C–H). The mobility of DNA was not affected by LMWC in the absence of oxidants (Lane I). Further increase in their concentration led to DNA condensation.

2.2. DNA condensation activity

DNA shows strong interactions with cationic cosolutes and this has both biological and technological significance. In our study we observed that LMWC in 10 mM PBS or water medium condenses or complexes with CTDNA in a ratio of approximately 1:1. No phase separation was observed till the DNA precipitation occurred with increase in LMWC concentration. The free energy of DNA condensation may vary with condensing ligand and solution conditions, thus DNA condensation is highly cooperative.[25] Electrostatic forces are equally important in such condensation. The laws of physics require that the strong repulsive interactions

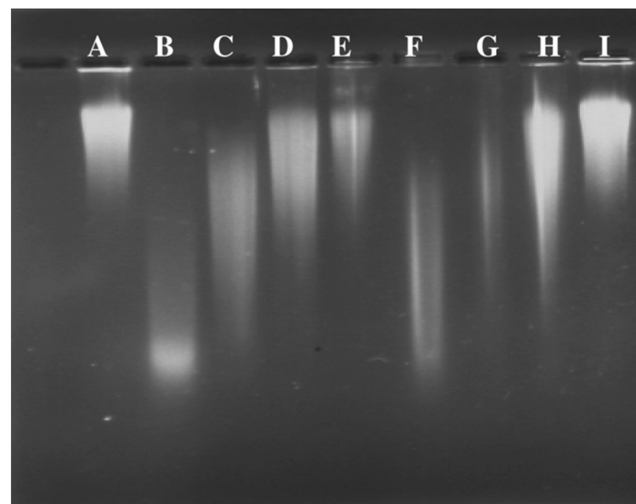

Figure 3. DNA protection by agarose gel electrophoresis: (A) native DNA, (B) oxidized DNA, (C)–(E) oxidized DNA protection by 2, 4 and 5 μg LMWC, respectively, (F)–(H) oxidized DNA protection by 5, 10 and 15 μg COs, respectively, (I) native DNA + LMWC (5 μg).

must be substantially neutralized as negatively charged DNA segments approach closely. Wilson and Bloomfield[26] observed that a critical concentration of ligand is required to effect DNA condensation, which increases with increasing salt concentration. Similarly, we observed that DNA condenses with LMWC and that the addition of NaCl can release DNA back into solution after being compacted with LMWC. DNA compaction could be reversed by 1 M NaCl.

In the process of condensation LMWC was found to bind to DNA. This was confirmed by showing the interaction of LMWC with ethidium bromide (EtBr) intercalated CTDNA, whose fluorescence intensity decreased as the concentration of LMWC increased (Fig. 4). Fluorometric assay can be employed if either the target compound or the product of reaction is fluorescent. In the current experiment, EtBr was used as a fluorescent dye, which binds to the double helical structure of DNA and gives the fluorescence signal. With 0.6 mL phosphate CTDNA and 250 μM EtBr/mL resulted in ~0.29 fluorescence intensity. The results showed that up to certain concentration of LMWC, there was a decrease in intensity from 0.299 to 0.137 and further increase in LMWC had no effect on fluorescence intensity. EtBr concentration exceeding more than 250 μM in the assay mixture had no effect on LMWC concentration. This suggests binding of LMWC in the minor groove and forming H-bonds to the backbone phosphates without distorting the double helix structure.

LMWC binding showed no significant dehydration or structural changes as revealed by circular dichroism (Fig. 5). The spectra taken for CTDNA (from 190 to 360 nm) and compared with LMWC binding till just below the critical DNA condensation showed only a little increase in ellipticity around 270 nm. Further, melting temperature curve for CTDNA in 10 mM PBS showed T_m at 57.7 °C, which was stabilized and shifted to 71.1 °C on the addition of 1 μg LMWC, thus preventing thermal denaturation (Fig. 6). The structure of condensed DNA was also investigated by TEM (data not shown). Although most macromolecules can be visualized with negative staining, DNA cannot be visualized by using uranyl acetate (1%). Since LMWC takes up the stain and when it intercalates with DNA, a single layer of 'toroidal' conformation can be viewed in TEM. It is already known that LMWC has the ability to complex DNA and protect against nuclease degradation.[27] The mechanism for this is not clear and deserves further investigation, but in all probability this LMWC/DNA condensation may be due to the electrostatic interaction.

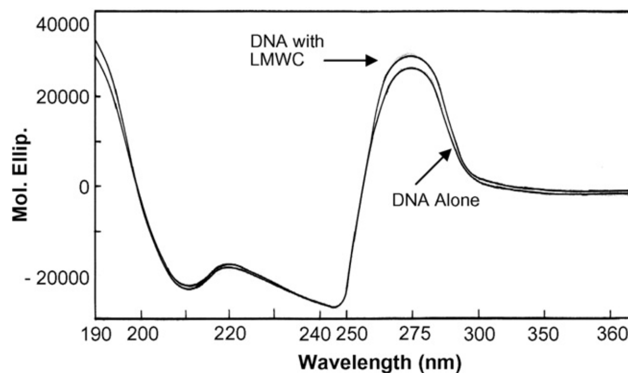

Figure 5. CD spectra of native DNA and that bound to LMWC.

This brings a new insight to the compaction/decompaction phenomena. Several interesting opportunities for applications like purification of DNA and controlled DNA delivery may emerge. Specifically for DNA delivery purposes and taking into consideration the toxicity factors, the behaviour of mixtures with biocompatible compounds like LMWC is worthy of further in depth investigation.

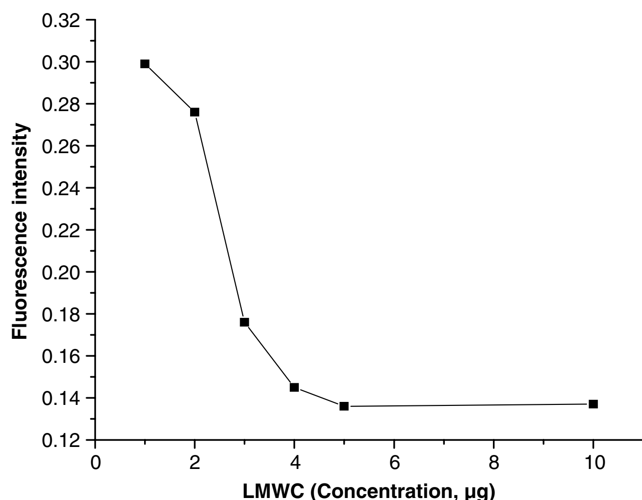

Figure 4. Fluorescence spectrum of DNA with EtBr and different concentrations of LMWC.

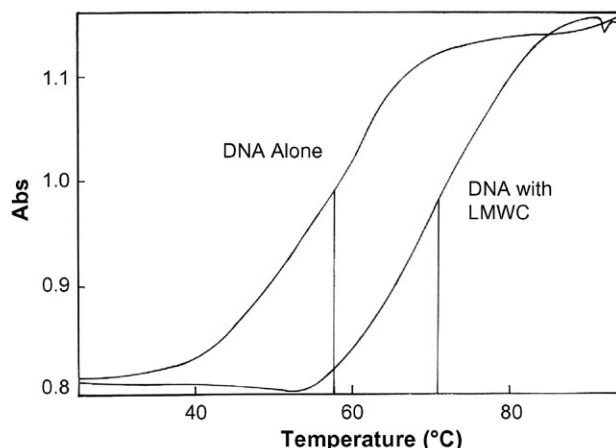

Figure 6. Melting curves of native DNA and that bound to LMWC, showing an increase in T_m due to binding.

3. Experimental

3.1. Materials

Unless stated otherwise, all materials and reagents were of highest analytical reagent grade.

3.2. Preparation of LMWC and COs

Briefly, chitosan (MW 96 kDa, degree of acetylation, DA ∼12%), obtained from heterogeneous de-N-acetylation of shrimp chitin,[30] procured from CFTRI Regional Center at Mangalore, India, solution (1% in 0.5% acetic acid), taken in a three-necked flat-bottomed flask, was purged with N_2 at 60 °C with stirring (200 rpm). Subsequently, potassium persulfate ($K_2S_2O_8$, 0.8 mM) was added and the depolymerization reaction was completed in 2 h. The reaction mixture was cooled to room temperature and later precipitated (LMWC) with alcohol (3 vol). It was dissolved in deionized water, dialyzed overnight and lyophilized. The alcoholic supernatant was concentrated by rotary flash evaporation, dialyzed and lyophilized to get COs. They were later characterized by IR, NMR and HPSEC.[9] LMWC was de-N-acetylated again by refluxing with 2 M NaOH at 100 °C for 2 h and alcohol precipitated to obtain a product of DA ∼1% and MW 40 kDa.

3.3. Hydroxyl radical scavenging

Hydroxyl radical scavenging activity of LMWC/COs (0.4–2 mg/1–8 mg, respectively) was measured according to the method given by Halliwell et al.[31] The thiobarbituric acid (TBA) adducts were produced by reacting 2-deoxy-D-ribose (2.8 mM) in a reaction mixture (1 mL) consisting of freshly prepared $FeCl_3$ (100 μM), EDTA (104 μM), H_2O_2 (1 mM) and ascorbate (100 μM) in 20 mM potassium phosphate buffer, pH 7.4. The reaction mixture was incubated for 1 h at 37 °C and subsequently heated in a boiling water bath for 15 min following the addition of 1 mL each of trichloroacetic acid (TCA, 10%) and TBA (0.5% in water). After cooling, the colour was measured at 535 nm. Curcumin (0.01–0.2 mg, Sigma, USA) was used as the reference standard.

3.4. Superoxide radical scavenging

Superoxide radical scavenging activity was carried out according to Gotoh and Niki[32] with minor modifications. Various concentrations of LMWC/COs (0.4–2 mg/1–8 mg, respectively) were added to the reaction mixture containing 100 μL of 30 mM EDTA (pH 7.4), 10 μL of 30 mM hypoxanthine in 50 mM NaOH and 200 μL of 1.42 mM nitroblue tetrazolium (NBT), and incubated at room temperature for 3 min followed by the addition of 100 μL of 0.5 U/mL xanthine oxidase and the final volume was brought up to 3 mL with 50 mM phosphate buffer (pH 7.4). The reaction was allowed to take place at room temperature for 20 min and the absorbance was measured at 560 nm.

3.5. DNA protection assay by agarose gel electrophoresis

The reaction mixture (20 μL) contained calf thymus DNA (CTDNA, 4 μg, Sigma, USA) in 20 mM PBS, pH 7.4 and different concentrations of LMWC (2, 4 and 5 μg) and COs (5, 10 and 15 μg) were added prior to $FeSO_4$ (1 mM) and ascorbic acid (10 mM) addition, and incubated for 90 min at 37 °C followed by the addition of loading buffer (xylene cynol 0.25%, bromophenol blue 0.25% and glycerol 30%) and the resulting mixture was transferred to 0.8% submarine agarose gel electrophoresis (50 V, 2 h).[27] The gel was stained with EtBr (0.05 μg/mL), destained in water and subjected to gel documentation.

3.6. Fluorescence measurements

Fluorescence measurements were carried out using a JASCO J77 spectrofluorimeter (JASCO, Easton, MD, USA). CTDNA (0.6 μM) and EtBr, 250 μM in 10 mM PBS or 5 mM HEPES buffer, pH 7.4 containing LMWC (of different concentrations) was divided into suitable aliquots to obtain subsequently 1 mL of final volume. The excitation at 540 nm and emission at 590 nm were measured. The changes in the emission intensity due to the binding of LMWC to DNA were measured after allowing the samples to stabilize for 5 min after each addition.[33] All binding experiments were carried out at room temperature and the fluorescence intensity values were corrected for dilution.

3.7. CD measurements

These were performed with a JASCO J-810 automatic recording spectropolarimeter (JASCO, Easton, MD, USA), calibrated with d-10 camphor sulfonic acid. Dry nitrogen gas was purged before and during the measurements. Slits were programmed to yield 10 Å band width at each wavelength (190–360 nm). The light path length of the cell used was 1 mm in the far UV and 10 mm in the near UV region and the measurements were made at 27 °C. The mean residual ellipticity was calculated using a value of mean residue weight from CTDNA.[33]

3.8. Melting temperature studies

The melting temperature (T_m) curves for CTDNA (0.6 μM) were determined in the presence or absence of LMWC (0.4 μM) in 10 mM PBS buffer. Absorbance

was recorded at different temperatures (20–95 °C, with a programme of 2 °C/min) using Ultraspec-4300 pro (Amersham Pharmacia-Biotech, Cambridge, England) spectrophotometer. T_m values were determined graphically from the percent hyperchromicity versus temperature plots. The precision in T_m values deduced from the variance in three repeated experiments was 0.05 °C.

3.9. Transmission electron microscopy (TEM)

The suspensions of condensed DNA with LMWC was applied to a carbon grid, stained with 1% uranyl acetate and viewed with a JEOL JEM-100S transmission electron microscope (JEOL, Tokyo, Japan) at a magnification of 10,000×.

4. Conclusions

In conclusion, LMWC and COs derived from $K_2S_2O_8$-induced chitosan depolymerization showed strong antioxidative effect, inhibiting to over 80% extent free radicals like $OH^•$ and superoxide anion and also offering protection to DNA from oxidative damage. LMWC showed reversible DNA condensation leading to stabilization with no change in its conformation.

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