

## Preparation of low-molecular-weight chitosan derivative zinc complexes and their effect on the growth of liver cancer cells in vitro\*

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**Abstract:** Low-molecular-weight (LMW) chitosan salicylaldehyde Schiff-base and its zinc(II) complexes were synthesized and characterized by Fourier transform-infrared (FT-IR) spectra, transmission electron microscopy (TEM), dynamic light scattering (DLS), gel permeation chromatography-multiangle laser light scattering (GPC-MALLS), and elemental analysis. The results of electrophoretic analysis suggest that the Zn complexes bound to DNA by means of electrostatic interactions and intercalation. The effect of the Zn complexes on the growth of SMMC-7721 liver cancer cells was investigated by sulforhodamine B assay in vitro. The results reveal that the growth of liver cancer cells was inhibited by LMW-chitosan and their Zn complexes. The inhibition rate of the Zn complexes was higher than that of LMW-chitosan ligand. The LMW-chitosan Schiff-base Zn complex exhibited higher anticancer activity than the LMW-chitosan Zn complex. Combining LMW-chitosan with Schiff-base and Zn improved its anticancer activity, which we ascribe to the synergistic effect between the chitosan matrix and the planar construction of the Zn complexes.

**Keywords:** anticancer activity; carbohydrate polymer; low-molecular-weight chitosan; Schiff-base metal complex; water-soluble.

### INTRODUCTION

Recent advances in understanding tumor biology have led to the discovery and development of novel anticancer drugs [1,2]. Although cisplatin is a widely used anticancer drug, it has a number of side effects and a limited spectrum of activity [3,4]. In order to reduce toxicity and widen the spectrum of activity, various platinum and non-platinum metal complexes have been prepared by varying the nature of the leaving groups and the carrier ligands [5–7]. Many natural antitumor products including polysaccharides have also been investigated [8–12]. Chitosan, the most abundant cationic polysaccharide in nature [13], is obtained by deacetylation of a widely distributed chitin. Due to non-immunogenicity, biodegradability, biocompatibility, and nontoxicity, chitosan and its derivatives have received much attention in the pharmaceuticals field [14]. They have been examined as potential materials for drug delivery [15], as gene therapy vectors [16], and as polymer-protein conjugates [17,18]. LMW-chitosan has

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high solubility in water and low viscosity and was shown to possess antifungal [19,20], antimicrobial [21], and antitumor activities [22,23]. And its antitumor activity depends not only on its chemical structure but also on the molecular size and charge properties [24–26].

Carbohydrates exert a wide range of functions in living organisms, and due to the wide distribution of metals and their complex functions for all forms of life, metal–carbohydrate interactions are essential to understanding bioinorganic chemistry [27,28]. Due to the manifold donor sites of nearly equivalent oxygen atoms and equilibria between several isomers, carbohydrates have been used as amine-substitute ligands [29–31]. The results of a previous study in which the interaction between LMW-chitosan Cu complexes and DNA was investigated reveal that these complexes exhibited excellent bioactivity [32].

Transition-metal complexes of Schiff-bases are becoming increasingly important as biochemical, analytical, antimicrobial, and anticancer agent reagents [33–38]. It is also well known that Zn is useful to reduce the cardio- and hepatotoxicity induced by some anticancer drugs [39]. In this paper, a new kind of LMW-chitosan Schiff-base Zn complex was prepared by combining LMW-chitosan salicylaldehyde Schiff-base, glycine, and Zn. The effect of the Zn complexes on the growth of the liver cancer cell line SMMC-7721 was investigated by sulforhodamine B assay *in vitro*.

## EXPERIMENTAL METHODS

### Materials

Chitosan powder with a degree of deacetylation approximately 90 % and a viscosity-average molecular weight  $2.0 \times 10^6$  was purchased from Zhejiang Yuhuan Biochemical Co. (China). LMW-chitosan (**1**) was prepared by the similar method of literature [32]. Ethidium bromide was obtained from Fluke. Agarose, pBR322DNA, RMPI1640, fetal bovine serum (not heat-inactivated), sulforhodamine B, and enzyme were commercially obtained from Sino-American Biotechnology Company (China). Other chemicals and reagents were commercially obtained.

### Instruments

The particle images were measured using transmission electron microscopy (TEM) (JEM-100SX, Japan). The particle size and its distribution were measured using dynamic light scattering (DLS) (Nano-ZS, Malvern, UK). The elemental content was determined by elemental analyzer (Vario-EL106, Germany). The zinc content was determined by atom absorption spectra analyzer (8410, Australia). Fourier transform-infrared (FT-IR) was measured by KBr disk on a FT-IR spectrophotometer (Alpha-Centauri FT-IR spectrophotometer, Japan). The molecular weight and distribution were determined by gel permeation chromatography (GPC) (Waters, USA)-multiangle laser light scattering (MALLS) (DAWN EOS, Wyatt, USA). Gel image manipulation was recorded with a gel image manipulation apparatus (DOC1000, USA).

### LMW-chitosan salicylaldehyde Schiff-base **2**

500 mg of LMW-chitosan **1** was added to 50 ml of methanol and the mixture was continuously stirred to ensure complete diffusion. Salicylaldehyde was added to the mixture at molar quantities equal to that of nitrogen in LMW-chitosan. The reactive mixture was stirred at room temperature for 16 h. The precipitate was filtered and extracted by Soxhlet with ethanol for 6 h. Finally, the crude product was filtered again and washed three times with anhydrous ethyl ether. After drying under vacuum, a light yellow powder, LMW-chitosan salicylaldehyde Schiff-base **2**, was obtained with a yield of 70 %.

### LMW-chitosan zinc complexes **3** and **4**

LMW-chitosan and  $\text{Zn}(\text{OAc})_2$  (molar ratio 1:1) were added in a solution of water/ethanol (V/V: 1/9). After stirring for 2 h at room temperature, the mixture was condensed by evaporation under reduced pressure. After adding anhydrous ethanol into the residue mixture the precipitate was obtained. It was mixed with glycine (at a molar ratio 1:1) in a solvent of water/ethanol and stirred for 6 h at room temperature. The product was filtered and washed with anhydrous ethanol three times. The LMW-chitosan zinc complex **3** was a yellow powder, yield 58 %.

The preparation of LMW-chitosan salicylaldehyde Schiff-base zinc complex **4** was similar to the preparation of complex **3**. It was also a yellow powder, yield 48 %.

### Sulforhodamine B assay

The liver cancer cell line SMMC-7721 was employed to evaluate the anticancer activity of the Zn complexes. The sulforhodamine B assay is a reliable and sensitive *in vitro* measure of drug-induced cytotoxicity [40]. In brief, the liver cancer cell lines were cultured in RPMI-1640 medium containing 5 % fetal bovine serum and incubated for 96 h under 5 %  $\text{CO}_2$  atmosphere at 37 °C. To obtain suspension in RPMI-1640 medium containing 5 % fetal bovine serum, an appropriate amount of enzyme was added to a culture bottle containing cell lines maintaining an exponential growth. After the number of cells was counted, the suspension cultures ( $2 \times 10^4$ – $5 \times 10^4$  cell/ml) were plated (100  $\mu\text{l}$ /well) in 96-well microtiter plates and incubated for 24 h under 5 %  $\text{CO}_2$  atmosphere at 37 °C. The tested compounds were diluted five gradients concentration and then added into each well (100  $\mu\text{l}$ /well) and incubated for 2–4 days under 5 %  $\text{CO}_2$  atmosphere at 37 °C. Cultures were fixed with trichloroacetic acid and stained for 10 min with 100  $\mu\text{l}$  of SRB solution in 1 % HOAc. Unbound dye was removed by washing five times with 1 % HOAc. The bound dye was extracted with 10 mmol/l unbuffered Tris base [tris (hydroxymethyl) aminomethane] for determination of optical density (OD) at 515 nm in a 96-well microtiter plate reader (Bio-Rad Laboratories model 550). The inhibition rate was calculated by the following formula:

$$\text{Inhibition rate}(\%) = [(\text{OD}^{\text{Control}} - \text{OD}^{\text{Test}}) / \text{OD}^{\text{Control}}] \times 100 \quad (1)$$

where  $\text{OD}^{\text{Control}}$  is the OD value at 515 nm of cells in the control plates and  $\text{OD}^{\text{Test}}$  is the OD value at 515 nm of cells in the test plates.

## RESULTS AND DISCUSSION

Chitosan nanoparticle drug carriers have been investigated in order to minimize side effects and enhance efficacy and water solubility of anticancer drugs [41,42]. Hence, LMW-chitosan **1** with a high solubility in water was prepared by depolymerization of chitosan. In order to improve coordinating ability, LMW-chitosan was modified by salicylaldehyde, which afforded LMW-chitosan salicylaldehyde Schiff-base **2**. Two kinds of LMW-chitosan zinc complexes **3** and **4** were prepared by coordination of LMW-chitosan **1** and **2** with glycine and Zn. They should exhibit the following advantages: they should increase the solubility of Schiff-base metal complexes without decreasing antitumor activity; the toxicity of Schiff-base complexes should be decreased after being supported in a natural polymer; and there should be a synergistic effect between the chitosan matrix and the planar construction of the Zn complex, which should enhance their anticancer activity.

## Characterization

### Particle sizes and distribution of compounds 1–4

The particle size of **1** was determined by TEM. It was observed clearly from TEM images (Fig. 1) that the particles were spherical in shape and exhibited some aggregation. The particle size distributions of **1–4** were measured by DLS. Mean particle sizes and polydispersity index (PDI) were calculated (Table 1). The size of **1** is 225 nm, which is similar to the result of TEM. The particle sizes of **3** and **4** were bigger than those of **1** and **2**, respectively. This may be due to the formation of partly coordinat- ing bonds between chitosan molecules and Zn.

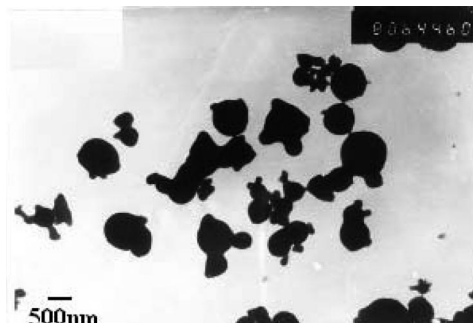


Fig. 1 TEM image of compound **1**.

Table 1 Mean particle sizes and PDI of compounds **1–4**.

Compounds	Size (d.nm)	PDI
<b>1</b>	224 ± 38 <sup>a</sup>	0.193 ± 0.061
<b>2</b>	240 ± 6	0.166 ± 0.028
<b>3</b>	436 ± 10	0.131 ± 0.084
<b>4</b>	339 ± 9	0.089 ± 0.076

<sup>a</sup>Each value is expressed as mean ± standard deviations (±S.D., *n* = 3).

### Structure and physicochemical characteristics of compounds 1–4

The data of elemental analysis follows (wt %): **1** (C: 37.11, H 6.90; N: 6.19); **2** (C: 35.07, H: 5.52, N: 4.82); **3** (C: 27.47, H: 5.93, N: 7.18, Zn: 15.67); **4** (C: 38.25, H: 5.93, N: 6.12, Zn: 13.11). As calculated from the above results, the substitution degree of amino group is 36 %.

In IR spectra, the characteristic absorption peaks of –O–H and –NH<sub>2</sub> in **1** appear at 3000–4000 cm<sup>-1</sup> in each compound (Fig. 2) [43]. The absorption at peak 1611 cm<sup>-1</sup> (N–H) becomes weaker and even disappears in **2**, and new peaks appear at 1631 cm<sup>-1</sup> (C=N) and near 1200 cm<sup>-1</sup> (Ph–OH) in **2**. Moreover, the characteristic absorption peak of phenyl appears at near 1450 and 750 cm<sup>-1</sup>. These results indicate that **2** is prepared by forming C=N groups at the N-position in chitosan [44]. Comparing **1** to **3**, the absorption peak (H–N) of the primary amine shifts from 1611 to 1631 cm<sup>-1</sup>, and the second hydroxyl absorption peak (–OH) of **1** shifts from 1075 to 1072 cm<sup>-1</sup>. The absorption peak (COO<sup>-</sup>) also appears at 1327 cm<sup>-1</sup> in glycine. The new absorption peak (O–Zn) and (N–Zn) appears at 518 cm<sup>-1</sup>. Comparing **2** to **4**, the absorption peak (C=N) shifts from 1631 to 1643 cm<sup>-1</sup>, and the new absorption peaks (COO<sup>-</sup>) in glycine, (O–Zn), and (N–Zn) appear at 1320, 518, and 467 cm<sup>-1</sup>, respectively. Moreover, the absorption peak (ph–OH) at about 1200 cm<sup>-1</sup> also shifts.

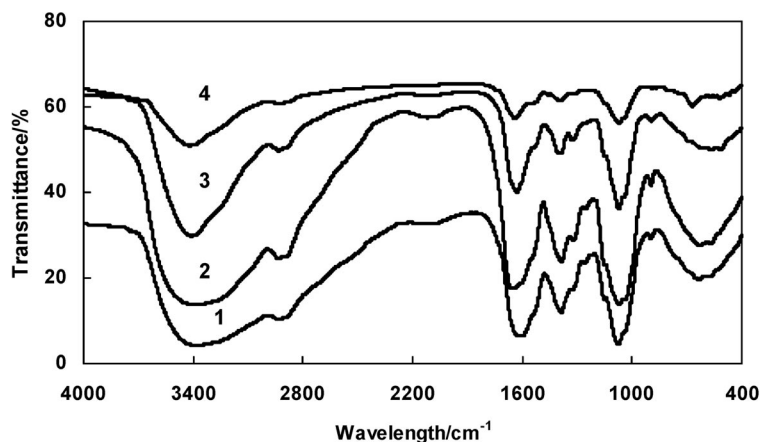
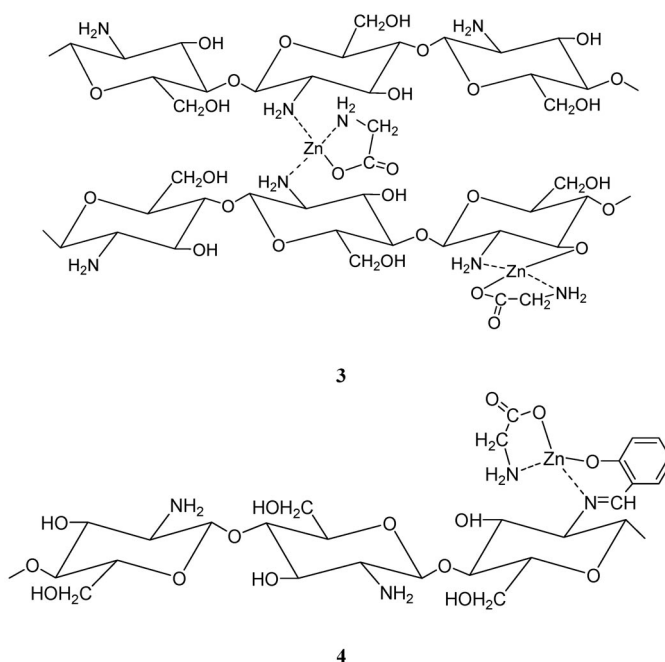


Fig. 2 Infrared spectra of compounds 1–4.

The number-average molecular weight ( $M_n$ ) of LMW-chitosan ligands (**1**, **2**) and their zinc complexes (**3**, **4**) were measured by GPC-MALLS. It was found that the  $M_n$  of LMW-chitosan ligands **1** and **2** is 2.2 and 2.7 kDa, respectively.  $M_n$  of LMW-chitosan zinc complexes **3** and **4** is 2.9 and 2.8 kDa, respectively. That means the molecular weight of **2** increased because the salicylaldehyde was bound to LMW-chitosan by C=N bond. The molecular weight of **3** increased because the Zn complexes were partly formed in different chains of **1**. Comparing with **2**, the molecular weight of **4** was not changed remarkably because the complexes were formed with Zn ion and Schiff-base on the chain of **2**.

Based on the results of elemental analysis, FT-IR and GPC, the amino groups of chitosan were substituted by Schiff-base groups. The structures of compound **3** and **4** were also suggested as Scheme 1. In compound **3**, the chitosan chain was partly cross-linked by coordinate bond. In compound



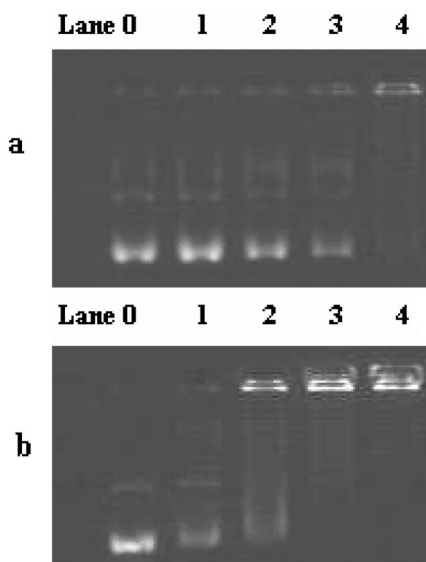
Scheme 1 Structure of compounds **3** and **4**.

**4**, the Zn complex was easily formed between LMW-chitosan Schiff-base ligand and Zn ion and glycine.

### Bioactivity studies

#### *Interaction of compounds 3 and 4 with DNA*

Interaction of **3** and **4** with DNA (pBR322DNA) was examined by electrophoresis using ethidium bromide as a probe. The reaction was performed in buffer solution (50 mmol/l tris-HCl, 0.2 mol/l NaCl, pH 7.5) for 12 h at room temperature. All samples were run on a 1 % agarose gel and stained with ethidium bromide to visualize DNA. As shown in Fig. 3, lane 0, a reference in this study reveals two DNA bands corresponding to the two forms of the plasmid DNA, i.e., supercoiled and nicked forms. Thus, the Zn complexes **3** and **4** showed excellent reactive activity with DNA. As the proportion of Zn complexes in the samples increased, the supercoiled DNA decreased as shown in lanes 1 through 4. This suggests that the Zn complexes bound to the DNA by means of intercalation and destroyed the secondary structure of DNA. There was also a corresponding increase in staining of DNA that did not migrate but rather remained at the top of the gel as shown in lane 4 (a) and lanes 3 and 4 (b). This indicates that the cationic complex electrostatically bound to the negatively charged phosphate backbone of DNA. This result suggests that there are both electrostatic and intercalation modes contributing to the interaction between the Zn complexes and DNA. It has been proved that the positively charged chitosan electrostatically bound to the negatively charged DNA [45]. Comparing **3** (a) with **4** (b), compound **4**'s electrostatic interaction with DNA was stronger than **3**'s. This reveals that these Zn complexes primarily interact with DNA via intercalation when the polymeric side chains were modified by the salicylaldehyde. This may be ascribed to the space effect of the large planar construction of **4**.



**Fig. 3** Electrophoretic mobility analysis of pBR322DNA interacting with Zn complex **3** (a) or **4** (b). Lane 0: only pBR322DNA (0.05 g/l). Lanes 1–4: pBR322DNA+ Zn complex **3** or **4** [0.05g/l + respective concentrations of the Zn complexes 0.5, 1.0, 2.0, 3.0  $\mu$ l ( $C_{Zn} = 1 \times 10^{-4}$  mol/l)].

### Inhibition of compounds 1–4 for liver cancer cell lines

The liver cancer cell line SMMC-7721 was employed to evaluate the anticancer activity of compounds 1–4. The results reveal that Zn complexes 3 and 4 inhibit the growth of these cells at a higher rate than 1 and 2, respectively (Fig. 4). With the increasing concentration of tested compounds, the inhibition rate was also increased. Compound 4 exhibited higher anticancer activity than 3. This behavior depended on the forming of Schiff-base on the polymeric side chains.

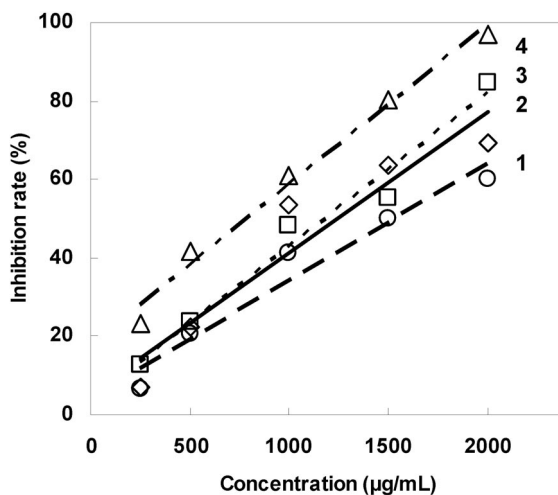


Fig. 4 Concentration–effect curves of tested compounds against the growth of liver cancer cell lines SMMC-7721.

In solution, the amino functions of chitosan are protonated and the resultant soluble polysaccharide is positively charged, whereas the cell surface is negatively charged. So the positively charged chitosan and Zn complexes can bind strongly to the negatively charged cell surface via electrostatic interactions. This interaction may result in adsorptive endocytosis and membrane instability [46]. Results of some related studies suggest that the observed antitumor activities were not due to direct killing of tumor cells, but might be due to increased production of lymphokines, leading to manifestation of antitumor effect through proliferation of cytolytic T-lymphocytes [23]. Furthermore, the large planar construction of the Zn complexes destroyed the secondary structure of DNA, and even inhibited the replication of DNA. Therefore, the synergistic effect between the chitosan matrix and the planar construction of the Zn complexes improved the anticancer activity of LMW-chitosan.

## CONCLUSION

Compounds 1 and 2 and their Zn complexes 3 and 4 with the second ligand (glycine) were prepared and characterized by FT-IR spectra, TEM, DLS, GPC-MALLS, and elemental analysis. These LMW-chitosan Zn complexes interacted electrostatically with DNA and intercalated between bases. All four of the compounds inhibited the growth of the SMMC-7721 liver cancer cell line. The inhibition rates of 3 and 4 against the growth of liver cancer cell lines were higher than those of 1 and 2, respectively. Compound 4 exhibited higher anticancer activity than 3. It was also found that the synergistic effect between the chitosan matrix and the planar construction of the Zn complex enhanced their anticancer activity. This class of LMW-chitosan Zn complexes should be further investigated as inhibitors of liver cancer cells.

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