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Preparation of chitosan–copper complexes and their antitumor activity

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Abstract—Copper complexes of chitosan have been synthesized. Complexes with different copper to chitosan ratios were tested in vitro as potential antitumor agents with 293 cells and HeLa cells. At the ratio of 0.11 mol copper per one chitosan residue, the complex exhibited a higher antitumor activity and less toxicity than other copper–chitosan complexes tested. In addition, this study showed that copper–chitosan complex inhibited tumor cell proliferation by arresting the cell cycle progression at the S phase in 293 cells.

Chitosan (CTS) is a high nitrogen-content biopolymer extracted from chitin. Chitosan contains multiple amino, hydroxyl, and acetamide groups and can form complexes with many metal ions.[1,2] The metal-chelating property of chitosan has been explored for different uses, but most previous studies on the chelation of copper ions with chitosan have been focused on its applications in adsorption,[3] metal ions separation, or waste water treatment.[4]

In recent years, various copper complexes have been tested for antibacterial[5] and antitumor properties. The copper complexes interact with DNA, leading to chemically induced cleavage of DNA and, thus, antitumor activity.[6–8] The mode of action is probably related to the binding of chitosan and copper, which is likely to leave some potential donor atoms free and these free donor atoms enhance the biological activity.[9,10] However, the high toxicity of the copper-loaded complexes prevents their medicinal use as an antitumor agent. Therefore, studies on reducing the toxicity and on better understanding the relationship between the complex structure and antitumor activity are of great interest. This study was undertaken to synthesize copper complexes at different metal to chitosan ratios in attempt to reduce toxicity and to investigate its possible activity as an antitumor agent.

Chitosan was prepared by N-deacetylation of chitin (supplied by Zhejiang Yuhuan Biochemistry Co. Ltd.) from shrimp shells according to a modified method based on a published procedure.[11]. Two hundred fifty milliliter of 60% NaOH was added into the reactor with 15 g chitin, then the mixture was refluxed at 150 °C for 0.5 h and cooled to room temperature, the upper liquid was removed. Then 200 mL of 60% NaOH was added, refluxed at 150 °C for 0.5 h, and cooled to room temperature. The sedimented product was filtered, collected, and washed with water until it is neutral, dried under infrared light, the chitosan weighing 11.06 g was obtained. Resulting chitosan was 81.2% deacetylated from the amino groups and has the average molecular weight of 780 kDa.[12] To prepare chitosan–copper at different ratios, 0.5 g chitosan was dissolved in 50 mL of 1% acetic acid solutions containing different amounts of copper sulfate at the following molar ratios of residue of chitosan to $CuSO_4{\cdot}5H_2O$: 1:1.56, 1:0.8, and 1:0.4. These complexes are designated as CTS–Cu (1), CTS–Cu (2), and CTS–Cu (3), respectively. The solutions were neutralized by diluted ammonium hydroxide and then stirred for three hours at 80 °C. After cooled to room temperature, the copper–chitosan complexes were precipitated by ethanol. The sedimented green product was filtered, washed several times with water, and dried under infrared light (Fig. 1).

Keywords: Copper complex of chitosan; Antitumor activity; Cell cycle.
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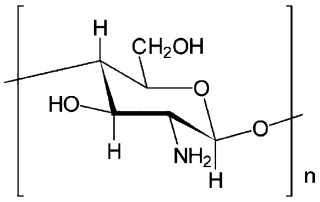

Figure 1. Structure of chitosan.

The formation of copper–chitosan complex was characterized by X-ray diffraction analysis, infrared spectra, and X-ray photoelectron spectra (XPS). Elemental analysis of the copper–chitosan (Table 1) showed that the amount of copper ions complexed with chitosan increased when increasing amounts of copper ions were added to the solution. Compared with the amount of the molar ratio of chitosan residue and copper ion calculated based on the elemental data, nevertheless, the actual amount of copper in each complex was lower than that added to the mixture, indicating that not all copper ions were complexed with chitosan.

The normal lung fibroblast cell line HLF, two tumor cell lines 293 and HeLa were obtained from Cell Bank of Chinese Academy of Science, Wuhan, China. Cells were plated in a 96-well microplate (100 μL/well) at a density of 1×10^5 per well. Background control wells contained the same volume of complete culture medium and were included in each assay. The microplate was incubated for 24 h at 37 °C. Then copper–chitosan complexes solved in 0.1 M HCL at various concentrations were added to the well and the plate was incubated for further 48 h. Cell proliferation assays were performed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan) to count living cells after adding 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo -phenyl)-2*H*-tetrazolium (WST-8) and 1-methoxyphenazine methosulfate (1-methoxy-PMS).[13]

The results showed that all of the copper complexes of chitosan inhibited the proliferation of the two tumor cell lines, HeLa and 293 at 10^{-3} mol/L, but not the normal human lung fibroblast cell line HLF (Table 2). Compared with Hela cell lines, the copper complexes of chitosan were found to be more selective to 293 cell lines in this experiment. The CTS–Cu(3) exhibited the highest antitumor activity, but chitosan alone was not effective. Heavy metal ions including copper ion have cytotoxicity to cell at 10^{-3} mol/L, so $CuSO_4$ exhibited antitumor activity at this level, but when copper ion was diluted to 10^{-4} mol/L, the percentage of inhibition decreased sharply to 3 or 5%.

The IC_{50} values of CTS–Cu(3) for 293 and HeLa cell lines were 0.34 and 0.48×10^{-3} mol/L, respectively. As shown in Table 3, CTS–Cu(3) has significant activity against tumor cell lines, it was more active than other copper complex of chitosan against tumor cell lines. Meanwhile, no copper complex of chitosan was found to have effect on the growth of normal cell line (HLF).

The highest antitumor activity exhibited by CTS–Cu(3) indicates that the antitumor activity is dependent on the concentration of copper in the complex of chitosan. The exact reason is unclear. One possible mode of action is that when chelated with copper ions, the positive charge on the amino group of chitosan was strengthened. As a result, the complex was easier to interact with anionic components of cell surface, increasing inhibitory activities.[14] More work is needed to confirm this hypothesis.

Table 1. Element analysis data of copper–chitosan complexes by a Carlo-Erha 1106 analyzer

Complex	Analysis found %				Mixture ratio	Calculated ratio
	Cu(II)	C	H	N		
CTS–Cu(1)	1.5	39.6	6.93	7	1:1.56	1:0.58
CTS–Cu(2)	3.4	34.2	5.79	6.1	1:0.8	1:0.3
CTS–Cu(3)	7.2	29.4	5.12	5.2	1:0.4	1:0.11

Table 2. Inhibition of cell proliferation by copper–chitosan complexes

Compound (mol/L)	Cell lines(%)								
	293			HeLa			HLF		
	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}
CTS–Cu(1)	88	26	8	51	19	3	23	10	2
CTS–Cu(2)	86	30	8	53	19	3	22	10	2
CTS–Cu(3)	92	48	13	71	30	5	28	14	3
CTS	28	3	3	22	5	na	8	na	na
$CuSO_4$	89	5	2	85	3	2	73	4	2

The cells were treated with copper complexes of different ratios for 48 h. Cell viability was determined by WST-8 and 1-methoxy PMS, and is presented as a percentage of living cells. Values are means ± SD from three separate cultures. The inhibition ratio was quantified by the formula: $IR\% = (C_0 - C/C_0)\ 100\%$, where C_0 = absorbance value from control group (without adding copper–chitosan complexes); C = absorbance value from copper–chitosan-treated cells.

Table 3. Cytotoxic activity of CTS–Cu(1), CTS–Cu(2) CTS–Cu(3), CTS against different cell lines

Cell line	Cytotoxicity (IC_{50}, μmol/L)				
	CTS–Cu(1)	CTS–Cu(2)	CTS–Cu(3)	5-FU	CTS
HLF	na	na	na	na	na
293	43 ± 2	40 ± 3	34 ± 2	41 ± 2	687 ± 22
HeLa	71 ± 3	63 ± 5	48 ± 4	42 ± 3	750 ± 41

Values are means of three experiments, standard deviation is given in parentheses (na = not active).

Table 4. Effects of copper–chitosan complexes on 293 cell cycle

Group	Cell cycle phase (%)		
	G_1	S	G_2/M
No copper complexes	59.8 ± 1.51	22.8 ± 1.11	17.4 ± 0.72
CTS–Cu(1)	51.8 ± 1.52	29.0 ± 1.21	19.2 ± 0.83
CTS–Cu(2)	52.7 ± 1.49	27.4 ± 1.09	19.9 ± 0.69
CTS–Cu(3)	44.7 ± 1.53	32.5 ± 1.31	22.8 ± 0.67

The 293 cells treated with different ratio complexes for 24 h. Values are means ± SD from three separate cultures.

Furthermore, flow cytometry analysis was employed to confirm that the copper complex of chitosan was required for the antiproliferative effect. The 293 cell lines were treated with the copper complexes of chitosan at different concentrations for 24 h. The cells were collected and suspended in the phosphate buffer salt solution (PBS). The suspension was centrifuged to collect the cells and fixed with 70% ethanol. The cells were resuspended in a solution containing RNase (50 mg/L) at 37 °C and propidium iodide (50 mg/L), respectively. The cell cycle distribution was measured by flow cytometry according to the manufacturer's instruction (Becton–Dickinson FACSort).

The effect of these compounds on the cell cycle of 293 cell lines was examined after the cell was treated with copper–chitosan complexes for 24 h at the 10^{-3} mol/L. In cells without adding copper–chitosan complexes, most of the 293 cells were at the G_1 phase, some at the S phase, and fewer at the G_2/M phase (Table 4). By comparison, the number of 293 cells increased at the S phase and decreased at the G_1 phase. All the compounds resulted in the arrest of the cell cycle progress at S phase. It is known that the checkpoint on cell cycle is the most important character at the S phase.[15] The checkpoint controls the progression of cell proliferation according to the environment signals and determines whether the cells proliferate or not.

In conclusion, the results of this study showed that chitosan can form stable complex with copper and different ratios of chitosan–copper complexes can be obtained by controlling the reagent ratios of complex reaction. The study also demonstrates that the copper complexes of chitosan have antitumor activity, while the sensitivities of tumor cell lines can differ, as indicated by the response of HeLa cells and 293 cells. With the three ratios tested, CTS–Cu(3) displayed a better efficacy in antitumor cell proliferation. CTS–Cu(3), which may represent a therapeutic potential of drug against cancer, was obtained. Furthermore, the data in the cell cycle also implied the same result. It is interesting to elucidate the detailed mechanisms of antitumor activity of this complex.

Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2006.04.077.

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