Anti-Hepatoma Activity of A New Selenium-Rich Chitosan from 6-Hydroxy Esterification of Chitosan Copper

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Abstract. This paper presents a technique to produce a selenium-rich chitosan by 6-hydroxy esterification of chitosan copper whose mole ratio of chitosan monomer and Cu is almost 1:1, which is suitable for the fields of food and feed chemistry or fine chemical technology, and belongs to the field of biomedical engineering technology. 6-Selenite chitosan copper will have better application prospect in medicine and make as selenium nutrient supplement in food, feed and agricultural fields, due to its higher selenium content and lower dosage than existing seleno-chitosan. The selenite chitosan copper effect than the common selenate chitosan. Selenite chitosan copper, which contains 10 times more selenium then existing seleno-chitosan or selenate chitosan, can greatly reduce its use and have a better anti-hepatoma effect. Metal ion protected amino group can bind to acid in the process of biological metabolism, and has a good targeting effect on the weak acidic environment of hepatocellular carcinoma tumors. Previous experiments in vitro showed that the IC50 value of selenite chitosan copper inhibiting HepG2 cells was 20.3 mg/L, and the inhibition rate to HepG2 cells was 87% at the concentration of 50 mg/L.

1. Introduction
With the improvement of life quality, the relationship between trace elements and health is paid more and more attention. Although contents of these trace elements in the body are very low, they are distributed in all tissues. If the body lacks these trace elements to a certain degree or contents of certain trace elements in the body are insufficient, a variety of diseases will attach the body and health quality will be seriously threatened. Among dozens of necessary trace elements in the body, zinc, copper and selenium, known as “three healthy treasures” are widely involved in the physiological process of human body and have great functions [1]. Among the three elements, selenium is more prominent and enjoys reputation of “Fire of Life”, “Guard Angle of Heart” and “King of Anti-cancer”. Medical studies have shown that low selenium intake is associated with heart and cellular diseases and with high incidence of breast, thyroid, prostate, lung, and colon cancers [2].
In 1973, WHO announced that selenium is an essential trace element for human body; in 1974, U.S. Food and Drug Administration provided that selenium must be added in edible animal feeds to ensure that people can get enough selenium; in 1988, Chinese Nutrition Society listed selenium as one of daily dietary nutrients. However, according to the Atlas of Endemic Diseases and Environmental Factors of the People's Republic of China, China is a large selenium-deficient country, with 700 million people living in selenium-deficient areas, resulting in a serious shortage of selenium intake per capita. Therefore, how to supplement selenium scientifically and orderly has become an important subject for nutritionists in China [3].

Selenium preparations for strengthening mainly include inorganic selenium preparations and organic selenium preparations [4], where inorganic selenium preparations mainly include sodium selenite and selenium dioxide; while organic selenium preparations mainly include selenoprotein (selenomethionine, selenocysteine, etc.), kappa-selenocarrageenan (selenium chitosan, seleno-k-carrageenan) and selenium-enriched yeast. Inorganic selenium has the advantages of high selenium content and low price, but absorption and utilization rate of inorganic selenium (such as Na$_2$SeO$_3$) is not ideal with low bioavailability, large toxicity. Besides, there is a very small gap between the poisoning amount and requisite amount; therefore, its use should be strictly limited. At present, the developed countries no longer use simple inorganic salt as a nutritional supplement of selenium, for example, Japan, the United States and other developed countries have banned the addition of Na$_2$SeO$_3$ and other inorganic selenium in food. Compared with inorganic selenium fortifier, organic selenium fortifier has less toxicity and higher absorption and bioavailability, besides, it can effectively store and accumulate selenium element and has wide biological activity with low selenium content, high price and large amount of selenium.

The study shows that organic selenium has a longer residence time in organism, and organic selenium can be stored in the condition of good selenium nutrition in the body, that when the selenium intake is insufficient, the stored organic selenium can be added to the physiological metabolism, so as to meet the demand of selenium. It will have great market potential if a mass product which can be taken daily without affecting sensory characteristics (such as, color, smell, taste) of the product according to characteristics of physicochemical properties of the selenium-enriched additives which are produced by effective method to transform biological organic selenium or a selenium supplement series drinks targeted to obvious selenium lack can be developed.

Chitosan is a kind of basic biological polysaccharide, which contains anti-tumor, hypolipidemic, anti-inflammatory, anti-bacterial and other biological activity; it is positively charged in acidic conditions, so it can neutralize by absorption discharge with anion on surfaces of cancer cells. It has better targeting, no toxicity, no other adverse reactions, and it is widely used in food additives [5-7].

Selenium chitosan is an organic selenium prepared by using chitosan and Na$_2$SeO$_3$ [8-10]. The results show that it can both inhibit proliferation and induce apoptosis in various tumor cells, including leukemia cells, and this inhibition effect is increased as time and dosage increase [11-13]. Further studies show that selenium chitosan can reduce contents of PML-RAR a fusion protein and cyclinDI protein in NB4 cells, inhibit the RaS signaling pathway and NF-KB signaling pathway in cells, increase the intracellular cAMP contents, reduce the contents of CGMP, increase the ratio of CAMP and CGMP and increases the intracellular calcium concentration to inhibit proliferation of NB4 cells of the acute promyelocytic leukemia cells and induce apoptosis; In addition, it is found from cell cycle analysis that chitosan could block cell cycle and stop it in the G0-G1 phase, suggesting that selenium chitosan has a good application prospect [11-13].

However, use of selenium chitosan is limited, due to the facts that selenium chitosan mainly depends on absorption of the amino to selenite ions in chitosan molecules, and the resulting product, not only has low content of selenium (0.4%) and its solubility is limited, but also reduces targeting of acidic microenvironment of tumor, resulting in too high IC$_{50}$ values in tumor cells (for example, IC$_{50}$ values in leukemia NB4 cells affected by selenium chitosan can be as high as 103.7mg/L, while IC$_{50}$ values in leukemia NB4 cells affected by Na$_2$SeO$_3$ is only 2.9mg/L) [12].

Studies have shown that chitosan metal chelates can significantly enhance antioxidant and antibacterial activity of chitosan, that calcium, iron, zinc or copper have similar absorption pathways
with selenium and are able to provide certain synergistic effects [1, 14, 15]. For this purpose, we propose for the first time the idea that synthesized selenium chitosan metal compound can be taken as selenium nutrient supplement by making use of advantages of the research group in synthesizing chitosan metal compound [16]. Through the shielding effect of metal ions, metal ions are introduced in chitosan 2-NH$_2$ and 3-OH for cyclization [16-18], while selenite esterification is carried out on 6-OH only, in order to obtain selenium chitosan metal complexes with high selenium content and high biological activity and maintain solubility of chitosan in inorganic acid and some organic acids water solution and its tumor targeting in acidic microenvironment.

Therefore, we propose to synthesize 6-selenite chitosan copper by 6-hydroxy esterification of chitosan copper [19] whose mole ratio of chitosan monomer and Cu is almost 1: 1 (see Figure 1), which is suitable for the fields of food and feed chemistry or fine chemical technology, and also belongs to the field of biomedical engineering technology.

![Chemical structure](image)

**Figure 1.** The step-by-step synthesis of 6-selenite chitosan copper from chitosan

2. Results and discussion

Comparing the IR spectra of chitosan copper chelate (synthesized by literature method) and 6-selenite chitosan copper, it can be seen that the strong double bond vibration peak [8-10] of selenite Se=O was observed at 713 cm$^{-1}$, the free hydroxyl peak disappeared, and there was no effect of free hydroxyl after the free hydroxyl was completely esterified. The peaks of the complexed hydroxyl and hydroxyl ions in the compound could clearly show (3502 cm$^{-1}$, 3190 cm$^{-1}$ and 2897 cm$^{-1}$), which confirmed that the selenium site of chitosan copper chelate was 6-hydroxy (see Figure 2). And it was also confirmed that the amino group of chitosan copper chelate did not take part in the deprotection reaction during the selenization reaction. These results further validate the structure of chitosan copper chelate esterified with 6-hydroxy.
Good inhibition of selenite chitosan copper on HepG2 cells has been showed in the anti-hepatoma activity tests in vitro, and gradually increased with the increase of time and dose (See Figure 3).

Compared with the existing selenate chitosan, selenite chitosan copper showed better inhibiting effect on HepG2 cells in vitro for 48 hours, and the dosage was reduced. The IC$_{50}$ value of selenite chitosan copper on HepG2 cells was 20.3 mg/L, and the inhibition rate was 87% at the concentration of 50 mg/L. The IC$_{50}$ value of selenate chitosan on HepG2 cells was 167.3 mg/L, and the inhibition rate was only 70% at the concentration of 800 mg/L [20]. The comparative chart of their effects are shown in the Figure 3.

Liver is a selenium pool of human body. If the selenium in liver is lacking, the activity of glutathione peroxidase in liver will be inhibited, and the liver will lose its physiological function, resulting in the accumulation of free radicals, lipid peroxides and other toxins in the body, resulting in metabolic disorders and imbalance of internal environment, leading to the occurrence of many diseases. It was
found that the inhibitory activity of selenite chitosan copper on HepG2 cells was significantly higher than that of selenate chitosan, and its potential anti-hepatocellular carcinoma value was self-evident. We suspect that the synergistic absorption of selenium by copper ions may promote the absorption of selenium by hepatocellular carcinoma cells, increase the activity of glutathione peroxidase in cells and inhibit the proliferation of hepatocellular carcinoma cells. At the same time, the introduction of copper ions protects amino groups and improves the targeting of drugs to the acidic microenvironment of hepatocellular carcinoma cells. Their further structure optimization, molecular design and structure-activity relationship were under way in our research group.

3. Experimental

Chitosan (C₆H₁₁O₄N·H₂O)ₙ (The degree of deacetylation is 99.9%); Water (Ultra pure); The other reagents are analytical pure; Chitosan copper (shortened as CTSCu; the mole ratio of chitosan monomer and Cu is almost 1: 1; the copper content is 20.65%) can be synthesized by document method [19].

3.1. Synthesis of 6-selenite chitosan copper (shortened as SeCTSCu) from Chitosan copper

Dissolve 3.0 g chitosan copper (chitosan monomer 10 mmol) in 200 ml 1% formic acid aqueous solution, then add selenious acid aqueous (10 mmol, containing 1.1 g SeO₂) directly. The reaction lasts for 2 hours at 75-85 °C until the selenite esterification was complete (there are no dissociated copper ions and incomplete selenous acid in the solution through ion detection). After cooling, add ethanol until no precipitation occurs, filter and wash precipitation 3 times with 75% ethanol, then dry to obtain 4.3g pure 6-selenite chitosan copper (bluish-green powder, yield 91%). Elemental Elemental Analysis for (C₁₂H₄₀Cu₂N₂O₂₆SSe₂)ₙ: Calculated. C, 15.24; H, 4.26; N, 2.96; Cu, 13.44; Se, 16.70. Found C, 15.11; H, 4.40; N, 2.86; Cu, 13.74; Se, 16.90.

3.2. MTT assay

Hepatocellular carcinoma cell line HepG2 was stem from affiliated Dongfeng Hospital, Chinese Medicine Experiment Center, Hubei University of Medicine. Culture medium and Fetal Bovine Serum (FBS, Gibco, USA). HepG2 cells grew in RPMI 1640 medium with 10% FBS and were cultured in an incubator with 37°C and 5% CO₂. After cell confluence reached 80% - 90%, the culture medium was removed and washed twice with phosphate buffer (PBS). The cells were digested by adding 3.0ml to 4.0 ml 0.25% trypsine ethylenediaminetetraacetic acid (EDTA) solution until the cell layer dispersed. Then add 5 to 6 ml of 10% (FBS) medium to stop digestion. The medium was moved into a centrifugal tube and centrifuged for 5 minutes. The medium was discarded and the cells were resuspended in fresh medium, and subsequent experiments were carried out. The cell proliferation assay was detected using MTT colorimetric assay. HepG2 cells were inculated in 96 -well at 37°C, 5% CO₂ for 24h, the volume of HepG2 cells was 200μl. Cells were treated with different concentrations of SeCTSCu and SeCTS (0, 25, 50, 100, 200, 400, 800mg/L), and incubated 24h or 48h. Then, washed with PBS, and added 100μl RPMI 1640 medium with 10μl MTT (stock solution of 5 mg/ml in water) to each well, and incubated
for another 4 h at 37°C. Next, took out the medium, added 150 ml DMSO to each well, and shaken the plate for 15 minutes. The absorbance of 490 nm (A490 nm) was determined by enzyme-labeled meter. Cell viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated control cells (Control).

4. Conclusion
Here we present a technique to produce a selenium-rich chitosan by 6-hydroxy esterification of chitosan copper, which is suitable for the fields of food and feed chemistry or fine chemical technology, and belongs to the field of biomedical engineering technology. 6-Selenite chitosan copper will have better application prospect in medicine and make as selenium nutrient supplement in food, feed and agricultural fields, due to its higher selenium content and lower dosage than existing seleno-chitosan.

In the process of complexation reaction, separating chitosan copper chelate [19] needs to consume a lot of ethanol to precipitate and wash. In the next step, it is very important to find the equilibrium point, which can ensure that no dissociation of copper ions and other side reactions occur in the esterification. When the selenious acid is not added directly but added dropping slowly, it is found that there are dissociated copper ions and incomplete selenious acid in the solution after 3 hours of reaction. Therefore, we will consider completing the complexation reaction of chitosan with copper ion and its 6-hydroxy esterification with selenious acid in one pot without separation of intermediates in late-stage studies.

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References

[1] Evans D M, Zhu G, et al. Genome-wide association study identifies loci affecting blood copper, selenium and zinc. *Hum. Mol. Gen.* 22 (2013) 3998-4006.

[2] Karam El-Bayoumy. 2010 The protective of selenium on genetic damage and cancer. *Mutation Research*, 457 (2010) 123-139.

[3] Xu R, Chen C, et al. Fingernail selenium levels in relation to the risk of obesity in chinese children: a cross-sectional study. *Medicine*, 97 (2018) e0027.

[4] Chen D, Du Y, et al. Research and Development Status of Domestic Selenium-enriched Foods and Analysis of Development Trend. *Journal of Trace Elements and Health in Chinese*, 31 (2014) 76-78.

[5] Zarzycki R, Modrzewska Z. 2003 Use of chitosan in medicine and biomedical engineering. *Polim. Med.* 33 (2003) 47-58.

[6] Dunia M. Garcia Cruz, Coutinho D F, Martinez E C, et al. Blending polysaccharides with biodegradable polymers. II. Structure and biological response of chitosan/polycaprolactone blends[J]. *J. Biomed. Mater. Res. B Appl. Biomater.* 87B (2010) 544-554.

[7] Liu Y, Wang H, Wu Z and Zeng X. Research progress of chitosan and its derivatives in biological medicine. *Hans J. Biomed.* 9 (2019) 89-95.

[8] Li Z, Lin M, et al. Preparation and Characterization of Chitosan Selenide. *Strait Pharm. J. Chin.* 17 (2005 (3)) 10-13.

[9] Miao J, and Li G, and Wang B. Study on the physical and chemical properties and the molecular structure of seleno-chitosan. *Chin. J. Marine Drugs*, 54 (2005 (4)) 124-126.

[10] Schrauzer G, and White D. 1983 Elemental selenium in organic selenium compounds their chemistry and biology. *Bioinorg. Chem.* 8 (1983) 303-305.
[11] Estevez H, Garcia-Lidon J C, Luque-Garcia J L, Camara C. 2014 Effects of chitosan-stabilized selenium nanoparticles on cell proliferation, apoptosis and cell cycle pattern in HepG2 cells: comparison with other selenospecies. *Colloids and Surfaces B: Biointerfaces*, 122 (2014) 184-93.

[12] Deng S, Zeng X, et al. 2010 Research on Acute Promyelocytic Leukemia Apoptosis Induced by Chitosan Selenide. *Guangdong Med. J.* 31 (2010 (06)) 678-80.

[13] Liu A, Song W, et al. Growth inhibition and apoptosis of human leukemia k562 cells induced by seleno-short-chain chitosan. *Methods & Findings in Experimental & Clinical Pharmacology*, 30 (2008) 181-6.

[14] Ghayourmobarhan M, Taylor A, et al. 2005 Determinants of serum copper, zinc and selenium in healthy subjects. Annals of Clinical Biochemistry, 42 (2005 (Pt 5)) 364.

[15] Cunha S D, Filho F M A, et al. 2003 Serum sample levels of selenium and copper in healthy volunteers living in Rio de Janeiro city. *Sci. Total Environ.* 301 (2003), 51-54.

[16] Zeng X, Wu Z, Liu Y, Wang H. Preparation and Application of Chelating Multi-metal Chitosan Selenite. *IOP Conf. Ser.: Earth Environ. Sci.* 185 (2018) 012018.

[17] Senlick S. Binding sites of Cu$^{2+}$ in chitin and chitosan. An electron spin resonance study. *Macromol.* 19 (1986), 192-195.

[18] Domard A. pH and c.d. measurements on a fully deacetylated chitosan: application to Cu$^{II}$-polymer interactions. *Int. J. Biol. Macromol.* 9 (1987), 98-104.

[19] Wang A, Shao S, Zhou J, Yu X. 2000 Synthesis and Characterization of Chitosan and Cu$^{II}$ Complex. *Acta Polym. Sin. Chin.* (2000 (3)) 297-300.

[20] Ji H, Feng Y, et al. Optimization of rolling flask culture technology for hepg2 cells and inhibitory effect of selenate chitosan. *Food Research & Development*, (2018 (3)), 234-238.