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Preparation of a Novel Thiolated Human-like Collagen Iron

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Abstract: In order to improve Human-like Collagen (HLC) iron binding capacity, the thiolated Human-Like collagen (SH-HLC) was adopted as chelating substrate. The binding of iron to SH-HLC was examined at varying pH, molar ratio (SH-HLC/Fe) and reaction time. The maximum binding capacity of SH-HLC was 31.18 mol iron per mol SH-HLC at pH 7.5, molar ratio 1:500 and reaction time 40 min. In addition, the SH-HLC and human-like collagen iron (SH-HLC-Fe) complex showed no toxicity to BHK cell through cell viability assay. These results suggest that the SH-HLC-Fe complex is a potential compound for use as a iron source.

Key words: Thiolated human-like collagen, iron

INTRODUCTION

Iron is considered to be one of the essential minerals required by living organisms and it is involved in major biological reactions such as energy metabolism, protein synthesis and enzymatic activity (Bothwell, 1995). Although with large amount in nature, iron deficiency affects approximately two billion people in the word. Iron deficiency can lead to significant physiological problems (Gillooly *et al*., 1983).

Previous studies in the literature have shown that protein with iron has been considered as a potential approach to delivering this nutritionally important mineral in required quantities to the consumer, thus helping in preventing iron deficiency (Nelson and Potter, 1979; Jacobs *et al*., 1989).

HLC is a recombinant collagen obtained from engineered Escherichia coli. It is a water-soluble recombined collagen with high availability, low pyrogen and similar structure to human collagen (Maeda *et al*., 1999). In the study, HLC was used as a good candidate. To improve its iron binding capacity, SH-HLC was adopted as chelating substrate(Strange *et al*., 1996).

The present study is to prepare the SH-HLC-Fe complex and optimize the synthesis condition of reaction pH, molar ratio (SH-HLC/Fe) and reaction time and then evaluate its iron binding capacity using flame atomic absorption spectrophotometer and its biocompatibility by cell viability assay. This work would provide some important theoretical evidences for the further study and application of chelate.

MATERIALS AND METHODS

Materials: The human-like collagen (HLC, patent number: ZL01106757.8, Mr = 97,000) was supplied by Xi'an Giant Biogene Technology Co. 3-(N-Morpholino) propanesulfonic acid (Mops), Ltd. S-acetylmercaptosuccinic anhydride (S-AMSA) and ferrous sulphate heptahydrate ($FeSO4 \cdot 7H2O$, Mr = 278.02) were purchased from Sigma Chemical Company. Cell Counting Kit-8 (CCK-8) was purchased from Signalway Antibody Co.Ltd. All other chemicals were of analytical grade and double distilled water was used throughout.

Preparation of SH-HLC: HLC was thiolated with S-AMSA according to the method of Klotz and RHeiney (1962) with minor modifications. The acetylthiol groups along with the negatively charged carboxyl groups were introduced to the HLC molecule. The modification was performed as follows: the freeze-dried native HLC was dissolved in 50 mM MOPs buffer (pH 8.0) at the concentration of 20 μM, S-AMSA was added as solid particles in small aliquots to the HLC solution under the constantly stirring to achieve desired weight ratio. The reaction was allowed to proceed for 1 h at room temperature following which, the SH-HLC molecules were separated from the unreacted S-AMSA on a Sephadex G-25 column. Finally, the SH-HLC were stored at 4°C after lyophilization for further use (Fan *et al*., 2012).

Effects of pH on the binding of iron to SH-HLC: The pH of 20 μM SH-HLC/HLC solution in MOPs buffer was adjusted over a range of 5.0 to 9.0 at room temperature.

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The ferrous sulphate heptahydrate solution (160 μL 500 mM) was added to the protein solution by gently stirring at room temperature for 1.5 h and then the solutions were filtrated through 0.45 μm filter, next through a gel chromatography to remove the free reagents. The purified chelate was finally freeze-dried and stored at 4°C. Finally, the iron content of chelate was determined by flame atomic absorption spectrophotometer (SOLAARM6, America).

Effects of ferrous ion concentration on the iron binding of SH-HLC: The SH-HLC solution (20 μM) was prepared in 0.5mM MOPs buffers (pH 7.0) at the molar ratio of SH-HLC/Fe^{2+} at 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, respectively. The solutions under gently stirring at room temperature for 1.5 h, the following procedure was the same as that mentioned above 2.2.

Effects of reaction time on the iron binding of SH-HLC: The SH-HLC solution was prepared at the molar ratio of SH-HLC/Fe^{2+} of 1:2000. The solutions were then stirring at 200 rpm at 25°C as increasing the reaction time form 20 to 80 min. The following procedure was the same as that mentioned above 2.2.

Cell viability assay: The lyophilized samples of native HLC, SH-HLC and SH-HLC-Fe were dissolved in RPMI-1640 medium with different concentration(5, 15, 25, and 35 μM) and then filtered through a 0.22 μm filter under sterilized conditions. Baby hamster kidney cells (BHK21) were cultured at a density of 1.0×10^4 cells mL^{-1} 96-well plates in a CO_2 (5%) incubator at 37°C. After incubation for 24 , different concentration samples were added to 96-well plates (100 samples μL $well^{-1}$). After incubation for 12 h and 24 h, 10 μL of CCK-8 was added to each well, after which the cultures were incubated at 37°C for an additional 4 h. Absorbency of the solution was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) Reader (MODEL550, Bio-Rad, USA). The relative cell growth (%) was calculated as:

$$\text{Relative cell growth} = \frac{A_s\text{-}A_b}{A_c\text{-}A_b}\times 100\%$$

where, A_s is the absorbency of sample, A_b is the absorbency of blank, A_c is the absorbency of control

RESULTS AND DISCUSSION

Effects of pH on the binding of iron to SH-HLC: The binding of iron to SH-HLC and native HLC as a function of pH was quantified and is displayed as binding isotherm curves (Fig. 1). As shown in Fig. 1, the amount of iron binding to SH-HLC were dependent on the pH in the range of 5.0-9.0. A small amount of iron binding could be observed between pH 5.0 - 6.0, followed by a sharp rise to maximum binding at pH 7.5, where 30 mol iron ions were bound to per mol SH-HLC. At low pH, besides the acid microenvironment affecting the release of protons in the binding process, there is a decrease in the ionization of the phosphate groups and this could cause a decrease in the cation binding of the modified protein. Less binding occurred above pH 7.5 and it was probably as a hydrogen ions compete with the iron ions for the binding sites (Chen and Fan, 2011). In our case, the maximum binding capacity of iron to the SH-HLC occurred at pH 7.5. In contrast, it can be seen from the curve of HLC that iron binding does not vary significantly with an increase in pH.The iron-binding quantity to native HLC, around 3 mol Fe moL^{-1} protein at different pH, is much lower than that of iron-binding SH-HLC.

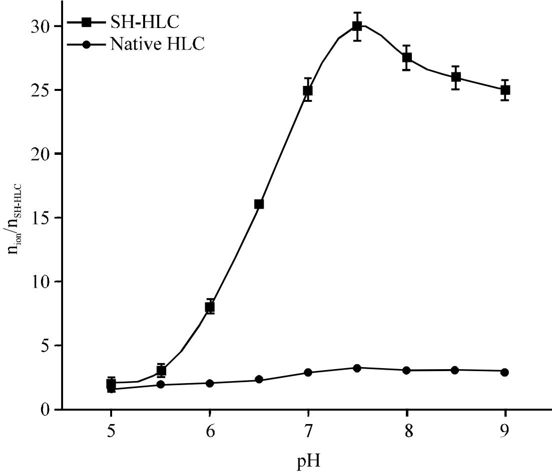

Fig. 1: Effects of pH on the binding of iron to SH-HLC/HLC

Effects of ferrous ion concentration on the iron binding of SH-HLC: The Fig. 2 demonstrates that the iron binding to SH-HLC increased from 18 to 28 mol as the molar ratio of SH-HLC/Fe increased from 1:100 to 1:500, whereas increase in the iron ion dosage from 1:500 to 1:700 lead to slightly decreased, it presumably precipitation of protein occurred at higher added iron concentrations. (Zanin *et al.*, 2012). At the optimum molar ratio (1:500), the amount of iron binding to SH-HLC reached 28 mol iron per mol SH-HLC, implying that the iron Fe-binding sites was reached saturation.

Table 1: Binding capacity for HLC and SH-HLC to iron

Samples	HLC	HLC-Fe	SH-HLC-Fe
Concentration of Fe (ppm)	0.00	1.75	17.46
Fe binding capacity n_{iron}/n_{HLC}	0.00	3.12	31.18

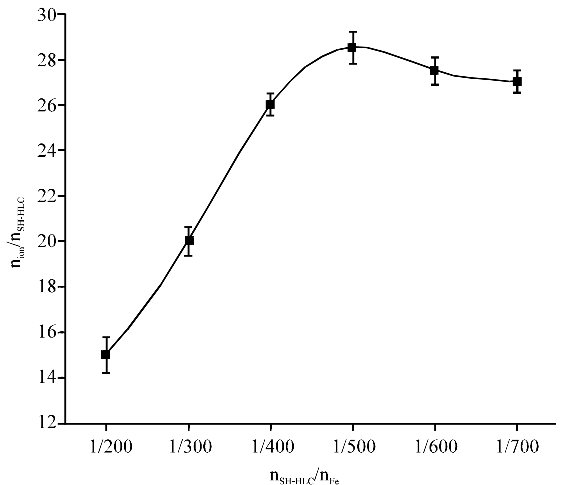

Fig. 2: Effect of SH-HLC-to-Fe molar ratio on the binding capacity of Fe

Effects of reaction time on the iron binding to SH-HLC: As illustrated in Fig. 3, the amount of iron binding to SH-HLC increased as the reaction duration was further increase from 20 to 40 min, indicating the positive effect of reaction time. Therefore, a reaction time of 40 min was considered as optimum for reaction condition.

Comparing the Fe-binding capacity of native HLC and SH-HLC: The HLC and SH-HLC solution were prepared in 0.5 mM MOPs buffers (pH 7.5) at the molar ratio of SH-HLC/Fe^{2+} at 1:500, respectively. The mixtures by gently stirring at room temperature for 40 min and then determine the total binding capacity of native HLC and SH-HLC by flame at Mic absorption spectrophotometer (Table 1).

As shown in the results, the binding quantity of iron to native HLC was much fewer than that of to SH-HLC, which due to the presence of acetylthiol groups and carboxyl groups on the exterior of SH-HLC allowed easy formation of the SH-HLC-Fe complex.

Cell viability assay: The WST-8 assay for cell viability was carried out with the cell counting kit solution (CCK-8) (Zhu *et al*., 2013). It is rapid and generally shows a good correlation with other viability tests and in vivo results.

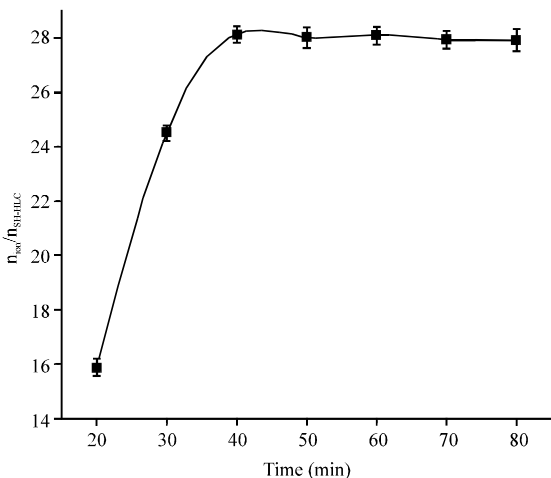

Fig. 3: Effects of reaction time on the iron binding to SH-HLC

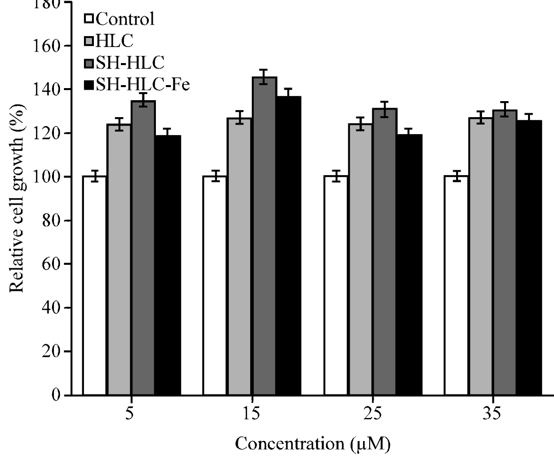

Fig. 4: Relative cell growth of HLC, SH-HLC and SH-HLC-Fe after culture for 12 h

The effect of HLC, SH-HLC and SH-HLC-Fe on BHK21 viability was determined by WST-8 assay. Native HLC could promote BHK21 proliferation (Zhu *et al*., 2013). Compared with the control, SH-HLC and SH-HLC-Fe triggered a significant increase in the number of BHK21 at 12 h (Fig. 4), especially at the concentration of 15 μM and the similar was true for 24 h (Fig. 5). Therefore, SH-HLC and SH-HLC-Fe were safe to BHK21 and could stimulate the normal growth of BHK21, which indicate that these new compound were potential to deliver iron to human beings at a required quantity so as to avoid the deficiency of iron.

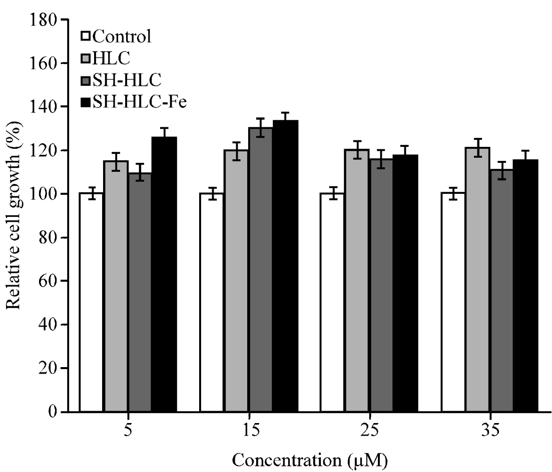

Fig. 5: Relative cell growth of HLC, SH-HLC and SH-HLC-Fe after culture for 24 h

CONCLUSION

In summary, the novel SH-HLC-Fe complex was successfully carried out using chelation reaction in 50 mM Mops buffer (pH 7.5). The maximum binding capacity of SH-HLC-Fe reached 31.18 mol Fe moL^{-1} SH-HLC by controlling the molar ratio of SH-HLC/Fe at 1:500 and reacting 40 min. Meanwhile, the binding quantity of iron to native HLC was much fewer than that of to SH-HLC. Besides, the SH-HLC and SH-HLC-Fe complex showed no toxicity to BHK cell through cell viability assay. All these results could pave the way for developing and applying the complex of SH-HLC-Fe.

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REFERENCES

Bothwell, T.H., 1995. Overview and mechanism of iron regulation. Nutr. Rev., 53: 237-245.

Chen, F. and D.D. Fan, 2011. Investigation on the Interaction between human-like collagen and Iron. Adv. Mater. Res., 287: 2941-2944.

Fan, D.D., C.H. Zhu, Y. Mi, Y. Sun and Y.H. Wang, 2012. A method for the introduction of acetylthiol groups into recombinant human-like collagen. Adv. Mater. Res., 535: 2416-2419.

Gillooly, M., T.H. Bothwell, J.D. Torrance, A.P. MacPhail and D.P. Derman *et al*., 1983. The effects of organic acids, phytates and polyphenols on the absorption of iron from vegetables. Br. J. Nutr., 49: 331-342.

Jacobs, D., G.D. Watt, R.B. Frankel and G.C. Papaefthymiou, 1989. Iron (+ 2) binding to apo and holo mammalian ferritin. Biochemistry, 28: 9216-9221.

Klotz, I.M. and R.E. Heiney, 1962. Introduction of sulfhydryl groups into proteins using acetylmercaptosuccinic anhydride. Arch. Biochem. Biophys., 96: 605-612.

Maeda, M., S. Tani, A. Sano and K. Fujioka, 1999. Microstructure and release characteristics of the minipellet, a collagen-based drug delivery system for controlled release of protein drugs. J. Controlled Release, 62: 313-324.

Nelson, K.J. and N.N. Potter, 1979. Iron binding by wheat gluten, soy isolate, zein, albumen and casein. J. Food Sci., 44: 104-107.

Strange, E.D., V.H. Holsinger and D.H. Kleyn, 1996. Rheological properties of thiolated and succinylated caseins. J. Agric. Food Chem., 44: 54-58.

Zanin, H., A.C. Peterlevitz, H.J. Ceragioli, A.A. Rodrigues, W.D. Belangero and V. Baranauskas, 2012. Magnetic and cytotoxic properties of hot-filament chemical vapour deposited diamond. Mater. Sci. Eng. C, 32: 2340-2343.

Zhu, C., Y. Sun, Y. Wang, Y. Luo and D. Fan, 2013. The preparation and characterization of noval human-like collagen metal chelates. Mater. Sci. Eng. C, 33: 2611-2619.