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Physicochemical Characteristics and Biological Activities of Chitosan-O-Kojic Acid Conjugate Polymer

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Abstract

The main objective of this work was to evaluate the physicochemical characteristics and biological activities of chitosan-o-kojic acid conjugate polymer (COS-O-KA) due to the conjugation of chitosan with kojic acid. The characteristics of water solubility, pH stability as well as hemolysis assay and cytotoxicity tests *in vitro*, zeta potential, and animal toxicity studies were investigated. The antioxidant activities *in vitro* were evaluated by DPPH•, •OH, $\bullet O_2^-$, $ABTS^{\bullet+}$, and Fe^{2+} chelating assays, which revealed that conjugation notably enhanced the antioxidant activities relative to free COS and KA. Owing to its non-cytotoxicity and low hemolysis, COS-O-KA might be considered a promising material for antioxidant applications.

Keywords: Chitosan oligosaccharide; Kojic acid; Antioxidant activity

Introduction

There are increasing evidence demonstrates that excessive amount of reactive oxygen species (ROS) and oxygen-derived free radicals (OFR) can destroy cellular components and may cause many diseases such as cancer, diabetes, aging, and atherosclerosis [1-4]. Hence, it is extremely important and necessary to develop and utilize safe and effective materials with antioxidant activity to prevent human body being damaged by free radicals [5,6]. Of late, numerous natural polysaccharides and their derivatives have been given evidence of displaying effective antioxidant activities and being developed as novel potential antioxidants [7-9]. Among various renewable natural polysaccharides, especially, chitosan oligosaccharide (COS), a promising and inexpensive resource for the fabrication of versatile biopolymeric material, is under intense research [10,11]. Due to its excellent anticancer, antimicrobial properties [12], intrinsic biodegradability, and biocompatibility, COS has been widely applied in several fields, such as pharmaceutical, biotechnology, cosmetics, textiles, effluent treatment, and agriculture [13-15]. Despite such desirable characteristics, its practical utility is limited due to the relatively lower antioxidant capacity compared with that of traditional antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) [16]. The presence of three useful active functional groups (a primary amine at C2, primary hydroxy at C6 and secondary hydroxy at C3 positions) in COS imparts unique chemical behavior and allows it to be readily modified to further augment the antioxidant activity [17-19]. Therefore, in recent years, research workers are continuing their unremitting efforts to design, prepare, and test the new, nontoxic, biocompatible, and low costing bioactive materials. The hydroxy groups of COS are the main sites for modification and a variety of 44 derivatives were synthesized through grafting reaction [20], quaternary reaction [21] and carboxymethyl reaction [22]. Among the methods of modification of COS, grafting reaction is one of the dramatic approaches, which can afford a great variety of monomers of active functional groups to COS chain by covalently bonded [23,24]. However, the issue of relatively low selectivity of some grafting COS-based antioxidant materials, leading to poor biocompatibility, cytotoxicity, and hemolysis, needs to be resolved. Kojic acid (KA, 2-hydroxymethyl-5-hydroxy-4H-pyran-4-one), a fungal metabolite, is frequently used as a browning inhibitor in the food industry

[25,26]. KA at concentrations in the range 1.6-50μg/mL did not effect, on the viability of normal skin cell and skin cancer cells during a 72-hours incubation [27]. Chlorokojic acid (Cl-KA) and further halo-derivatives of KA, which act as efficient antioxidants and as ligands for nucleophilic and electrophilic substitutions, have been synthesized and applied in food, medical, cosmetic, and chemical industries [28,29]. Besides, they are well-known as potent bidentate metal chelators [30]. Partially, kojic acid-chitosan- tri-polyphosphate nanoparticles and kojic acid-loaded chitosan coated magnetic nanoparticles (KA-CS-MNPs) enhanced the antibacterial activity of KA against both Gram-positive and Gram-negative, and showed good bio adhesive properties [31,32]. Inspired by biological activity of COS and KA, we developed a simple approach for preparing COS-O-KA polymer, and COS-O-KA was prepared based on our previously reported process [13]. The reaction conditions, yield, degree of substitution (DS), number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity index (PID) were shown in Table 1. Furthermore, the water solubility, pH stability, cytotoxicity tests *in vitro*, zeta potential, antioxidant activity and animal toxicity were systematically evaluated. Figure 1 shows the reaction scheme for the preparation of COS-O-KA and its bioactivity. We postulated that the combination of COS and KA, with synergistically diverse modes of action, could facilitate further derivatization to achieve excellent antioxidant efficiency and low cytotoxicity (Table 1) (Figure 1).

Table 1: Reaction conditions for COS with KA, Yield, DS, Water solubility, Mn, Mw, and PID for the COS-O-KA1-3.

Sample	Reaction conditions[1]	Yield (%)	DS[2]	Mn[3]	Mw[4]	PID[5]
KA				-	-	-
COS				8837	9271	1.04
COS-O-KA1	-5 ºC /2.16:1/5h	78	1.41±0.30	7520	8365	1.11
COS-O-KA2	-10 ºC /1.07:1/4h	72	1.22±0.40	7617	8978	1.17
COS-O-KA3	25 ºC /1.07:1/4h	60	0.98±0.50	7721	85111	1.1

Reaction temperature/Molar ratio (Chlorokojic acid to N-benzylidene COS)/Reaction time.
Values are expressed as mean±standard deviation.
Mn: number-average molecular weight.
Mw: weight-average molecular weight.
PID: polydispersity index.
-: is no testing.

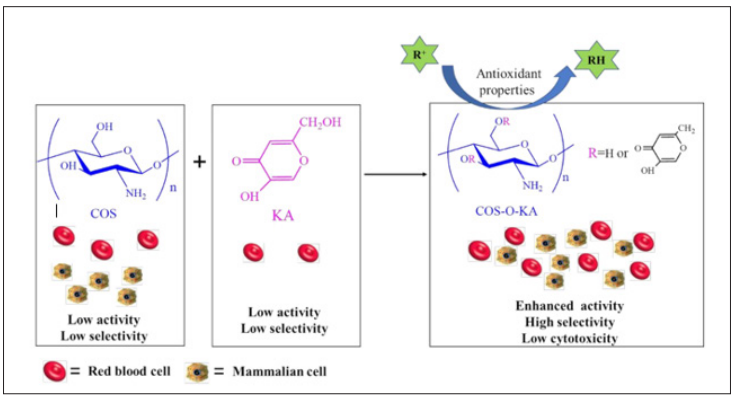

Figure 1: Reaction scheme for the preparation of COS-O-KA and its bioactivity.

Materials and Methods

Materials

COS (8828 Da; 90% deacetylation degree) was sourced from Zhejiang Jinke Biochemistry Co.Ltd, (Zhejiang, China). COS-O-KA was prepared based on our previously reported process [13]. KA was obtained from Wuhan Weisheng Biochemical Co. Ltd., (Wuhan, China). Cl-KA was prepared based on the Uher's technique with minor modifications [33]. COS-O-KA was prepared based on our previously reported process. DS was defined as the mol ratio of bonded kojic acid per mol of glucosamine calculated from the original mass of COS. The deacetylation degree (DD) and DS were calculated from the integrals of 1H NMR spectrum. The average molecular weight (Mw) of COS and COS-O-KA1-3 were calculated using gel permeation chromatography (GPC). 2,20-Azinobis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was purchased from Honeywell International Inc, USA. Nitro blue tetrazolium (NBT) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Human aortic smooth muscle cells (HASMC CC-2571) was purchased from Saiqi Biological Engineering Co. Ltd., (Shanghai, China). Riboflavin, methionine, Tris-HCl buffer solution (0.05 mol/L Tris, 0.10 mol/L NaCl, pH=7.4), N-methyl piperazine, benzaldehyde, pyridine, potassium ferricyanide, ethylene diamine tetraacetic acid (EDTA),

N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), thionyl chloride (SOCl2), phosphate-buffered saline (PBS), acetone, ethanol, methanol, and deuterium oxide (D2O) were purchased from Sinopharm Chemical Reagent Co., Ltd., (Shanghai, China). All other chemicals and reagents were of analytical grade.

Determination of pH stability

Five g of the samples (COS, COS-O-KA1, COS-O-KA2, and COS-O-KA3) were dissolved in 1% acetic acid (100 mL). The COS-O-KA1-3 were prepared under different reaction conditions, and with different DS, Mn, and PID, which shown in Table 1. One M NaOH solution was added gradually to the solutions. Determination of pH stability of the solutions were examined by transmittance at 600nm by using UV1000 UV-Vis spectrophotometer (Techcomp Ltd., China). [34,35].

In vitro hemolysis assay

In brief, 10mL of freshly collected blood from a 26-year old healthy male donor, who gave signed informed consent, was centrifuged at 580×g for 10min. The separated erythrocytes (RBC) were diluted to 5% (v/v) after washing thrice with Tris (10mM) and Tris buffer (150mM, pH = 7.2). Several concentrations (0.5-2.5mg/mL) of sample solutions were added into the RBC stock (1:1, v:v) in 96-well microplates, followed by incubation in a shaking incubator at 37 °C for 1h. Subsequent to centrifugation for 10min at 180×g, the supernatants (100μL) were added to fresh microplates and mixed with Tris buffer (100μL). Triton X-100 (0.1% in Tris buffer) which is able to lyse RBC completely was used as positive control, while Tris buffer was used as negative control. The hemolysis (%) was estimated by absorbance at 541nm with a Spectra Max M5 microplate reader (Molecular Devices, Pennsylvania, USA) using the equation:

$$\text{Hemolysis (\%)} = [(A_s - A_b)/(A_t - A_b)] \times 100\% \quad \text{Eq(2)}$$

Where A_s, A_t and A_b are the absorbance value for the samples (COS, KA, and COS-O-KA1-3), positive control (Triton X-100) and negative control (Tris buffer), respectively. The procedures were carried out in accordance with the protocols of Jiangnan Univerisity's Institutional Medical Ethics and Use Committee (JN. No20190228c0360425[8]).

In vitro biocompatibility assay

The cell viability was investigated through 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) protocol [36]. Human aortic smooth muscle cells (HASMC CC-2571) were added to a 96-well plate (1 ×105 cells/well), diverse concentrations of COS-O-KA1-3 (5,10,15,20 and 25mg/mL) 200μL were added and were incubated at 37 °C for specific times. Subsequently, MTT (20μL) was added to the wells, followed by incubation for 4h, and centrifugation at 720×g for 5min. Next, the supernatant was replaced with DMSO (100μL), the plate was shaken for 15min and the absorbance was recorded at 490nm.

Zeta potential measurement

Zeta potential was determined for all the samples (COS,KA and CO-O-KA1-3) at pH 2,3,4,5,6,7,8,9,10,11 and 12 by means of a Zeta PALS Analyzer (Brookhaven Instruments Corp., Austin, Texas, USA). The calibration of instrument was done with a standard of –50±5 mV at 25 °C according to the operation instructions.

DPPH• scavenging activity

Briefly, different concentrations of COS, KA, and CO-O-KA1-3 (0.5-2.5mg/mL) were transferred to 96-well plates (50μL/well) and DPPH (200μL, 0.4mM) was supplemented to the wells [37]. The mixtures were vigorously shaken and subsequently maintained in the dark for 30min. Absorbance at 516nm was recorded. The DPPH• scavenging capacity (SA) was estimated using Eq. (3):

$$\text{SA (\%)} = [1-(A_1-A_2)/A_0] \times 100 \quad \text{Eq. (3)}$$

Where A_0 (water instead of sample), A_1, and A_2 are the absorbances of the control, sample, and background (water in place of DPPH), respectively.

OH• scavenging activity

The •OH scavenging activity of antioxidants were estimated according to an earlier method [5]. The mixture containing 1mL COS, KA, and CO-O-KA1-3 (0.5-2.5mg/mL of samples in distilled water), 2mL $FeSO_4$ (9mM), and 2mL H_2O_2 (0.3%) in salicylic acid-ethanol solution was incubated at 37 °C for 30min, and subsequently cooled down to measure their absorbance at 510nm against reagent blank. The •OH scavenging activity (SA) was measured using Eq. (4).

$$\text{SA (\%)} = [1-(A_1-A_2)/A_0] \times 100 \quad \text{Eq. (4)}$$

Where A_0, A_1, and A_2 are the absorbances of the control, sample, and background, respectively.

$\bullet O_2^-$ scavenging activity

Reaction was accomplished in a mixture containing sodium phosphate buffer (50mM, pH 7.8), NBT (70μM), methionine (13mM), EDTA (100mM), riboflavin (10Mm), and antioxidant samples (1 mL,0.25-2.5mg/mL) that according to an earlier method [3]. The absorbance was recorded at 560nm after incubated at room temperature for 10min. The $\bullet O_2^-$scavenging activity (SA) was measured using Eq. (5).

$$\text{SA (\%)} = [1-(A_1-A_2)/A_0] \times 100 \quad \text{Eq. (5)}$$

Where A_0, A_1, and A_2 are the absorbances of the control, sample, and background, respectively.

ABTS•+ scavenging activity

The ABTS$\bullet^+$ scavenging capacity was determined according to an earlier method [24,38], with some modification. Briefly, ABTS (7mM) was reacted with potassium persulfate (4.95mM) and the mixture was kept in the dark at 25 °C for 12h. Then, each sample (0.5-2.5mg/mL of samples in distilled water) and ABTS working solution (200μL) were mixed together, and the absorbance was monitored at 735nm. The ABTS$\bullet^+$ scavenging capacity (SA) was measured using Eq. (6).

$$\text{SA (\%)} = [1-(A_1-A_2)/A_0] \times 100 \quad \text{Eq. (6)}$$

Where A_0, A_1, and A_2 are the absorbances of the control, sample, and background, respectively.

Fe2+ chelating activity

The Fe^{2+} chelating capacities of COS, KA, and CO-O-KA1-3 were determined by means of Topal's technique with some modifications [4]. Initially, ferrozine solution (0.2mL, 5mM), $FeCl_2$ solution (0.05 mL, 2mM), water (2.75mL) and samples (1.0mL, 0.5-2.5mg/mL) were mixed together. After 10min incubation at 25 °C, UV-Vis spectra were recorded at 562nm. The chelating capacity (CA) was measured using Eq. (7).

$$CA\ (\%) = [1-(A_1-A_2)/A_0] \times 100 \quad \text{Eq. (7)}$$

where A_0, A_1, and A_2 are the absorbances of the control, sample, and sample with deionized water instead of $FeCl_2$ solution, respectively.

Animal toxicity study

Forty healthy female SLAC KM mice (6-8weeks old, 20.0±2.0 g) were randomly segregated into three drug administration groups and a control group, with 10 mice in each group. COS-O-KA1-3 solution in sterile PBS (100μL) was injected through the tail vein to the drug administration groups at the doses of 25,18 and 6mg/kg in the high, medium and low dose groups, respectively. The treated mice were monitored 7 days after administration. The control mice were intravenously administered with 9mg/mL NaCl solution. The mice were handled in accordance with Jiangnan Univerisity's Institutional Animal Care and Use Committee protocols (JN. No20190228c0360425[8]).

Results and Discussion

Determination of pH stability

Figure 2a shows the stability of all the samples at different pH values. Increasing the pH of aqueous solution from 3.0 to 10.0, COS-O-KA1 exhibited the highest solution stability at higher pH values (pH=10, 86.94%). As the pH was increased from 7.0 to 9.0, the transmittance of the COS solution decreased rapidly. On the contrary, the transmittance of COS-O-KA1-3 solutions slowly declined. These results signified that COS-O-KA1-3 polymer had superior pH stability than COS in basic conditions (Figure 2).

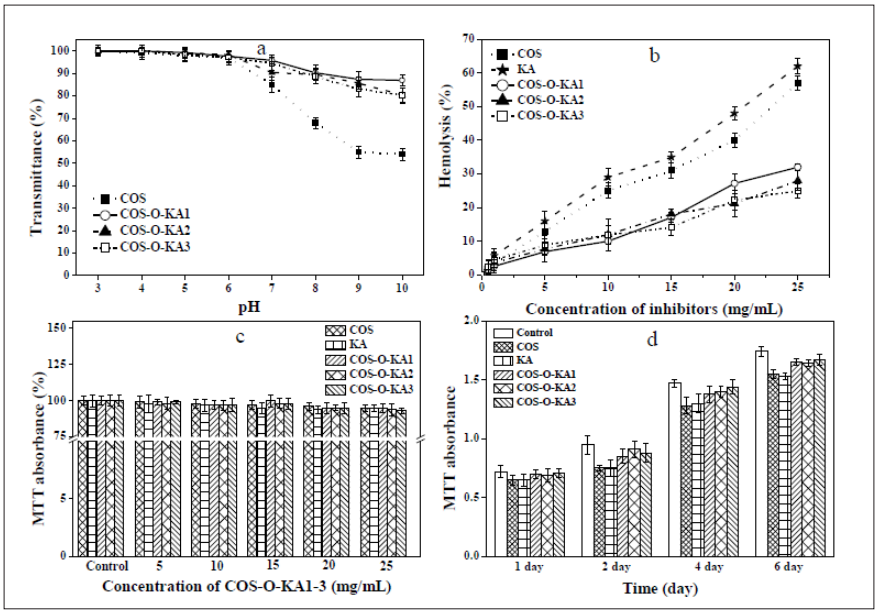

Figure 2: a: pH stability of COS and COS-O-KA1-3; b: Hemolytic activity and of COS, KA, and COS-O-KA1-3 at different concentrations. c: MTT of HASMC cultured in a series of concentrations of COS-O-KA1-3 for 1h. d: HASMCs cultured with 15mg/mL COS-O-KA1-3 from 1 to 6 days.

In vitro hemolysis assay

At 15mg/mL, COS-O-KA1-3 exhibited less than 20% hemolytic percentage, whereas the hemolytic activities of COS and KA were more than 30% at the same concentrations (Figure 2b). At 25mg/mL, the hemolytic activity was less than 35% for COS-O-KA1-3, while the activities of COS and KA were greater than 50%. The percentages of COS-O-KA1, COS-O-KA2, and COS-O-KA3 ranged from 25% to 32% with slightly increased hemolytic activity with increasing DS. Hence, comparing COS with COS-O-KA1-3, grafting with KA reduced the hemolytic activity and COS-O-KA1-3 shows excellent non-hemolysis as well as potent antimicrobial and antioxidant activity [13, 39]. These results signified an exceptionally high selectivity of COS-O-KA1-3 for microbes over human RBC.

In vitro biocompatibility study

In the MTT assay, HASMC were viable in the presence of diverse concentrations of COS-O-KA1- 3 for 1h and there was no decline in HASMC viability below the concentration of 25mg/mL (Figure 2c). As shown in Figure 2d, the MTT absorbance increased with time, indicative of cell proliferation in the presence of 15mg/mL COS-O-KA1-3. This was similar to the results of the control treatment from days 1 to 6, signifying the *in vitro* biocompatibility of COS-O-KA1-3.

Zeta potential measurement

The surface charge of COS-O-KA1-3 was denoted by zeta potential (Figure 3a). At acidic pH, COS-O-KA became polycationic and more protonated amine groups were formed as the DS of the

COS-O-KA reduced, resulting in a higher zeta potential [40]. It was confirmed that COS-O-KA3 with least DS of 0.98 displayed maximum charge density (33.33mV). At alkaline pH, the molecules were deprotonated with negative surface charge and were aggregated through intramolecular hydrogen bonding. Thus, DS altered the pKa of COS, protonating COS-O-KA1-3 differently from COS under identical pH conditions.

DPPH• scavenging activity

The DPPH• scavenging capacities of the samples increased in a concentration-dependent mode (Figure 3b). The DPPH• scavenging capacities of 2.5mg/mL of COS, KA, COS-O-KA1, COS-O-KA2, and COS-O-KA3 were 52.28%, 59.33%, 85.21%, 87.35%, and 82.83%, respectively. The relatively superior scavenging ability of COS-O-KA1-3 could be ascribed to the existence of active amine and 5-hydroxypyranone groups in the structure. We have noticed that the order of scavenging activities of COS derivatives was as follows: COS-O-KA2 (DS = 1.22±0.4) > COS-O-KA1(DS = 1.41±0.3) >COS-O-KA3 (0.98±0.5). This result indicated that COS derivatives, which has the medium DS was showed the strongest DPPH• scavenging activities. Hence, we assumed that when the γ-pyranone structure that contains an enolic hydroxyl group, active hydroxyl, and active amino of COS-O-KA get to the right ratio [41], the COS-O-KA could show the strongest DPPH• scavenging activity [42].

OH• scavenging activity

Figure 3c shows the COS, KA, and COS-O-KA1-3 scavenge •OH generated by the Fenton reaction. The •OH scavenging activity was increased obviously with the increased concentration of samples. The percentage scavenging effect of COS-O-KA1-3 ranged from 43.32-78.12%, 50.31-81.35%, 45.31-80.64%, respectively, which was higher than that of COS (23.30-53.28%) and KA (35.50-65.53%), at concentrations of 0.5-2.5 mg/mL. It proved that COS-O-KA1-3 had stronger •OH scavenging ability than COS and KA. The probable mechanisms of the scavenging activity of COS-O-KA1-3 against •OH may be as followings [5,15]: (1) The enolic hydroxyl groups of γ-pyranone structure and the hydroxyl groups of COS skeleton in the COS-O-KA polymer can react with •OH by the typical H-abstraction reaction. (2) The residual free amino groups ($-NH_2$) of COS-O-KA1-3 polymer can react with •OH. (3) The protonated $-NH_2$ groups (NH_3^+) can also react with •OH in solution through addition reaction (Figure 3).

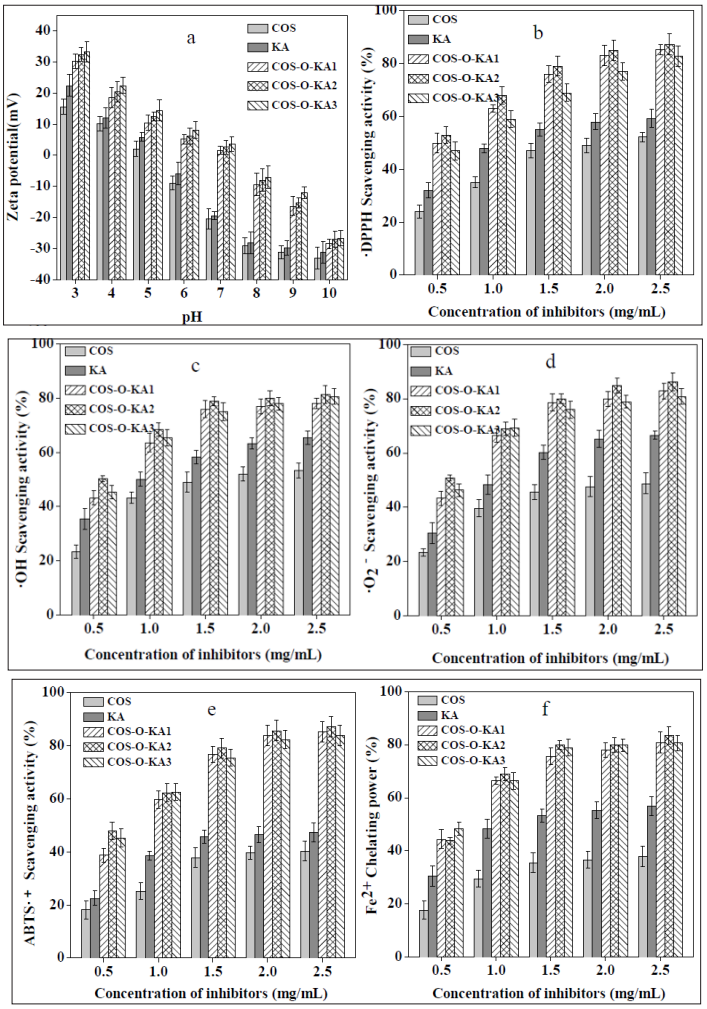

Figure 3: a: Zeta potential of COS, KA, and COS-O-KA1-3(1mg/mL) in different pH. Scavenging activities on DPPH•(b), •OH(c), $•O_2^-$(d), ABTS•$^+$(e), and Fe^{2+} (f) chelating power.

•O2– scavenging activity

The samples exhibited remarkable $\bullet O_2^-$ scavenging activity, which correlated significantly with the increase in the concentrations ($P<0.05$) (Figure 3d). At 2.5mg/mL, the scavenging abilities of COS-O-KA1-3 were above 80%. Nevertheless, the scavenging activity of COS and KA were much lower than COS-O-KA1-3, with the scavenging activity of 48.74% and 66.68% at 2.5mg/mL, respectively. Based on the free-radical theory, the COS-O-KA1, COS-O-KA2, and COS-O-KA3, which have alcohol or phenolic hydroxyl groups or amino groups in their molecular chain, can react with free radicals to yield stable macroradicals [5]. Thus, the active hydroxy and amine groups in COS-O-KA played main role in scavenging $\bullet O_2^-$ [24].

ABTS•+ scavenging activity

Our results revealed that the ABTS$\bullet^+$ scavenging activities of the samples shared similar trends that increased slowly with the increased concentration, and with the similar results in the DPPH• assay (Figure 3e). It is notable that the scavenging activities of COS-O-KA1-3 against ABTS$\bullet^+$ were increased slowly (0.5-2.5mg/mL) and reached the plateau at 2.0mg/mL (83.87%, 85.76%, 82.33%, respectively), which was much greater than that of COS (39.65%), and KA (46.54%). Hence, we assume that the phenolic hydroxyl groups in the COS-O-KA polymer play a vital role in the hydrogen- and electron-donating ability [38].

Assay of Fe2+ chelating activity

The Fe^{2+} chelating capacity is an indicator of antioxidant potency since the antioxidants are capable of hindering the formation of red Fe2+-ferrozine complex [2]. The results are summarized in Figure 3f. The Fe^{2+} chelating capacities of the samples were all concentration dependent. In particular, COS-O-KA1-3 displayed higher Fe^{2+} activity than COS and KA. The chelation efficiencies for 2.5mg/mL of COS, KA, COA-O-KA1, COA-O-KA2, and COA-O-KA3 were 37.85%, 56.79%, 80.88%, 83.52% and 80.65%, respectively. It was demonstrated earlier that the molecules that contain two or more of the -OH, -SH, -COOH, $-PO_3H_2$, C=O, $-NR_2$, -S-, and -O- functionalities, generally display metal-chelating activities [43]. The structures of COS and KA include β-1,4-linked glucosamine (-OH, C=O, $-NR_2$, and -O-) and 5-hydroxypyranone, respectively. Consequently, the chelating capacity of COS-O-KA was attributed to its distinctive catechol fragment and the numbers and positions of the functional groups. Grafting catechol group onto COS resulted in an enhanced Fe^{2+} chelating capacity and offered a route for the development of novel COS-based polymers for chelation [24,44].

Animal toxicity study

Animal toxicity studies of COS-O-KA1-3 were also tested conducted using healthy female SLACKM mice (6-8 weeks old and weighing 20.0±2.0 g) by tail vein injection at one control group and three groups for drug administration (6, 18, and 25mg/kg, respectively). After 7 days, the mice in the three drug administered groups did not develop any clinical signs of toxicity even at 25mg/kg dose. The weight of each group increased gradually as did that of the control group (Table 2). Visual inspection of the animals did not reveal any illness and no mortality occurred. Therefore, the *in vitro* and in vivo studies established the non-/low-toxicity of COS-O-KA1-3 (Table 2).

Table 2: Weight changes of mice in each group (X±S, n=10).

Drug dose (mg/kg)	0 d	1 d	3 d	5 d	7 d
Control	25.3±1.15	26.1±1.34	26.7±1.07	27.2±1.34	28.3±1.19
COS-O-KA1					
6	25.7±1.02	26.5±1.08	26.9±2.11	27.8±1.65	28.8±2.07
18	25.1±1.36	26.3±1.26	26.7±1.24	27.5±2.02	28.5±1.14
25	25.8±2.01	26.7±1.25	27.4±1.33	27.8±1.21	28.6±1.01
COS-O-KA2					
6	25.5±2.27	26.3±1.38	26.8±3.14	27.6±2.18	28.7±1.23
18	25.4±1.87	25.9±1.78	26.5±2.24	27.3±1.02	28.2±1.43
25	25.2±0.98	25.7±2.15	26.2±1.72	27.5±3.13	28.6±0.83
COS-O-KA3					
6	25.5±1.39	25.8±3.01	26.5±0.98	27.7±1.54	28.3±3.15
18	25.6±1.12	26.0±2.11	26.8±0.75	27.4±2.04	28.8±2.01
25	25.5±2.01	26.2±3.21	26.5±2.31	27.9±3.25	28.3±3.15

Conclusion

It was demonstrated that COS-O-KA polymer exhibited outstanding scavenging capacities against DPPH•, •OH, $•O_2^-$, ABTS•$^+$, and Fe^{2+} chelating power, all of which were superior to those of native COS and KA. These are possibly due to the active hydroxy and amine groups in the COS-O-KA chains, which play a vital role in the free radical scavenging. Furthermore, the non-cytotoxic nature and biocompatibility of COS-O-KA afford opportunities for the future applications of this antioxidant material (Figure 1).

Author Contributions

Xiaoli Liu designed the study, collected test data, interpreted the results and drafted the manuscript. 273 Wancui Xie and Xihong Yang completed the statistical analysis of the data. Xiaobei Zhan and 274 Wenshui Xia participated in its design and revised the manuscript.

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