Carbohydrate Polymers 101 (2014) 776–785

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effects of sulfate group in red seaweed polysaccharides on anticoagulant activity and cytotoxicity

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ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form 4 August 2013
Accepted 3 October 2013
Available online 12 October 2013

Keywords:
Agarose
Carrageenan
Sulfation
Oligosaccharide
Anticoagulant
Cytotoxicity

ABSTRACT

In this paper, the structural effects of two main red seaweed polysaccharides (agarose and carrageenan) and their sulfated derivatives on the anticoagulant activity and cytotoxicity were investigated. The substitution position rather than the substitution degree of sulfate groups shows the biggest impact on both the anticoagulant activity and the cell proliferation. Among them, C-2 of 3,6-anhydro-α-D-Gal*p* is the most favorable position for substitution, whereas C-6 of β-D-Gal*p* is the most disadvantageous. Moreover, the secondary structures of glycans also play a key role in biological activities. These demonstrations warrant that the red seaweed polysaccharides should be seriously considered in biomedical applications after carefully tailoring the sulfate groups.

1. Introduction

As an important portion of polysaccharides, red seaweed polysaccharides have attracted much attentions because of their biological activities and abundant marine storage. Among them, agarose and carrageenan are two easily available ones which have been widely used as gelling and thickening agents (Jiao, Yu, Zhang, & Ewart, 2011). Their structures are both linear, with alternating α-(l-3) and β-(l-4) linkages situated between galactose or glactose analogs. The structural differences appear in the stereo configuration of 4-linked α-galactose residues (D in carrageenan and L in agarose) (Rochas and Lahaye, 1989). Their sulfated derivatives have also been found to possess unique anticoagulant, antioxidative, antitumor and antiviral functions (Chen, Wu, & Wen, 2008; Jiao et al., 2011; Mayer, Rodriguez, Berlinck, & Hamann, 2009; Pomin & Mourao, 2008; Yoshida et al., 1988). Sulfate groups can be located on C-2, C-4, and/or C-6 of D-galactose, for instance, natural sulfated agar was found to be substituted on C-6 and C-2 of β-D-galactopyranose (Gal*p*, symbolized as G) unit (Goncalves et al., 2005; Jiao et al., 2011). The most commonly used carrageenans are normally classified as κ, ι and λ forms according to their sulfation patterns and the existence of 3, 6-anhydro-α-D-galactopyranose (3,6-AG, symbolized as DA) on D-units (Shanmugam & Mody, 2000). As shown in Fig. 1, ideally, in the three forms of carrageenans, the number of sulfate groups in every disaccharide unit is 1, 2, and 3, respectively, and in agarose, no sulfate group exists. Compared with the κ- form, the ι-carrageenan has an additional sulfate group on C-2 of the DA residue, resulting in two sulfates per disaccharide unit. Meanwhile, each disaccharide unit of λ-carrageenan has three sulfate groups on C-2 of G residue, C-2 and C-6 of the 4-linked residue (α-1, 4-G), and with little or no 3, 6-anhydride bridge (Jiao et al., 2011). But commercially, the sulfate content of κ-, ι-, and λ-carrageenan is only 15–20%, 28–30% and 32–39%; while the 3,6-AG content is 28–35%, 25–30%, and 0–10%, respectively (Usov, 2011).

Many researches have been published concerning the relationship between the structures and the biological activities of sulfated red algal galactans (Toida, Amornrut, & Linhardt, 2003; Usov, 1992). But the results were mainly based on data from oligosaccharides. In most cases, sulfated polysaccharides with high-molecular-weight from natural source or after chemical modification were degraded by acid hydrolysis, methanolysis, partial reductive hydrolysis, or enzymolysis, and their structures were analyzed primarily relying on 1D or 2D ^{1}H and ^{13}C NMR spectroscopy, as well as electrospray ionization-mass spectrometry (ESIMS) (Gonçalves, Ducatti, Duarte, & Noseda, 2002; Goncalves et al., 2005; Pavlenko, Belogortseva, Kalinovskii, & Ovodov, 1976; Yu et al., 2002). Considering that the hydrolytic treatment might bring about changes in the secondary structure, and at the same time, the conformations of the whole polymer chains might also have synergistic effect on biological activities, a clear analysis on the chemical structure of pristine polysaccharides with high molecular weight is necessary. However, there have been some but still few reports on this issue owing to the difficulties in structural analysis of complex conformations (Opoku, Qiu, & Doctor, 2006; Yuan et al., 2005, Araújo, Noseda,

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http://dx.doi.org/10.1016/j.carbpol.2013.10.010

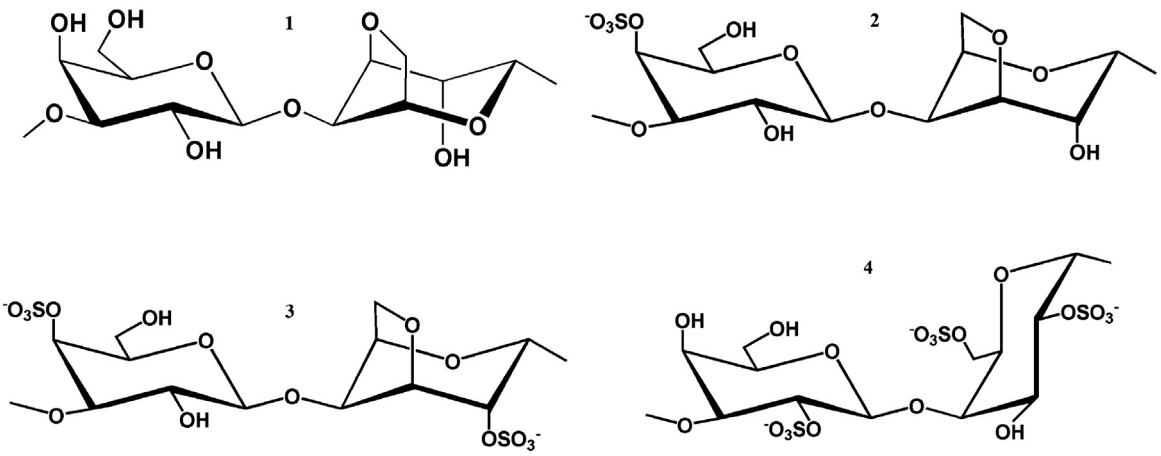

Fig. 1. Chemical structure of (1) agarose, (2) κ-, (3) ι- and (4) λ- carrageenan.

Cipriani, & Goncalves, 2013). In these researches, additionally, the sulfation pattern and the molecular weight of glycans have been revealed as two important factors on their biological activities (Bao, Duan, Fang, & Fang, 2001; Haroun-Bouhedja, Ellouali, Sinquin, & Boisson-Vidal, 2000; Nishino & Nagumo, 1991, 1992). Meanwhile, many recent evidences indicated that besides charge density arose from sulfate groups, some structural effects which were stereospecific could also be important to biological activity, for example, the anticoagulant activity could be influenced by the glycosidic linkage (either (1 → 3) or (1 → 4)) and the neighboring sulfate groups (Chaidedgumjorn et al., 2002; Pereira, Melo, & Mourão, 2002). Nevertheless, the existing researches on various factors were discrete, so comprehensive explorations combining these factors together remain to be investigated. Consequently, to carry out analyses based on intact structures and multifactor influences, we here prepared sulfated agarose, sulfated κ-carrageenan, desulfated κ-carrageenan, and κ-carrageenan oligosaccharide, respectively, and evaluated their anticoagulant activities and cytotoxicities, so that hopefully find the relationship between D-galactose structures and their bioactivities.

2. Materials and methods

2.1. Materials

κ-Carrageenan (B.R.) was purchased from Weijia (Guangzhou, China). ι-carrageenan (commercial grade, C1138), λ-carrageenan (low substitution degree, commercial grade, 22049, Lot No. 1408463), and 1,2,4,5-pyromellitic acid (A.R.) were purchased from Sigma–Aldrich (USA). Agarose (B.R.) was purchased from Genebase (Shanghai, China). Chlorosulfonic acid (A.R.) was purchased from Xihua (Tianjing, China). Heparin sodium standard (197 IU/mg) was purchased from Hengyuan Qitian (Beijing, China). 1640 culture medium, fetal bovine serum (FBS), and double resistant were purchased from Gibico (USA). All other chemical reagents used, including KCl, 30% H_2O_2, absolute EtOH (without dehydration), $NaBH_4$, phenol, $BaCl_2$, D-galactose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and isopropanol, were of analytical grade. Some anhydrous organic solvents, including pyridine, formamide, dimethyl formamide (DMF), and dimethylsulfoxide (DMSO) were also of analytical grade and dehydrated through routine approaches before use.

2.2. Purification, degradation and classification of κ-carrageenan

The κ-carrageenan was purified by KCl precipitation method (S.F-Tischer et al., 2006). κ-Carrageenan was firstly dissolved in H_2O at 75 °C to form 0.5% aqueous solution. The solution was quickly filtered before cooling down. Then equal volume of 8% KCl solution was added by stirring for 3 h. The resulting mixture was kept to rest overnight at room temperature. After discarding the supernatant by filtration, the insoluble part was dialyzed (cut-off 3500) for 3d, and freeze-dried.

The purified κ-carrageenan was degraded through ultrasonic-assisted oxidative degradation (Chen et al., 2011). κ-Carrageenan was dissolved in water at 75 °C by stirring to form 2% aqueous solution. The solution was cooled down to 50 °C, and a certain volume of 30% H_2O_2 was added dropwise under ultrasonic condition. After the degradation was completed, 20 wt% $NaBH_4$ was added, and kept to rest with pH > 10 for 2 h under room temperature.

The reduced κ-carrageenan was concentrated by rotary evaporator for 0.5 h, precipitated by triple volume of EtOH, classified with different dialysis bags (cut-off 3500∼12,000) for 3d, and freeze-dried to give degraded samples with different molecular weights. The resulting degraded carrageenan was dissolved in water and separated by centrifugal ultrafiltration (cut-off 3000) repeatedly. The solution containing carrageenan with $M_w \approx 3000$ was dialyzed (cut-off 1000) for 3d and freeze-dried to give κ-carrageenan oligosaccharide ($M_w \approx 3000$).

2.3. Preparation of sulfated agarose, κ-carrageenan, and desulfurated κ-carrageenan

The agarose and κ-carrageenan were sulfated by pyridine-chlorosulfonic acid method (Jie, Zhang, Chen, Mao, & Tang, 2012). Chlorosulfonic acid was slowly added dropwise into anhydrous pyridine with vigorous stirring in ice bath to form the SO_3-pyridine complex. After the reaction was completed, the formamide solution containing 1.5% carrageenan or agarose was added under higher temperature. The pH of the resulting solution was adjusted to slightly alkaline with 2.5 mol/L NaOH. Then the solution was concentrated, precipitated by triple volumes of EtOH, and centrifuged. The precipitate was dialyzed (cut-off 3500), washed and centrifuged for multiple times, and freeze-dried.

The κ-carrageenan was desulfurated through a mild desulfurization method (Miller & Blunt, 1998). κ-Carrageenan (0.5 g) was dissolved in anhydrous DMSO (100 mL). Then 15 (v/v) % pyridine, 0.06 mol 1,2,4,5-pyromellitic acid and 0.024 mol NaF was added in one portion at 100 °C by stirring for several hours. The resulting solution was neutralized to pH 7–8 with 2.5 mol/L NaOH solution. Then the product was precipitated by triple volume of EtOH,

washed and centrifuged for multiple times, dialyzed (cut-off 3500) and freeze-dried.

2.4. Characterization of agarose, carrageenan and their derivatives

2.4.1. Molecular weight

The intrinsic viscosity (η) and viscosity-average molecular weight (M_v) were determined by the viscometric method. 0.7% Na_2SO_4 aqueous solution was used as the solvent, and the temperature was maintained at 35 ± 0.1 °C. The molecular weights of the standard carrageenan samples were 3.81×10^5 and 1.70×10^4, and the calculated parameters were: $K = 0.0043$, $\alpha = 0.88$.

The weight-average molecular weight (M_w) and the polydispersity index (PDI) were determined on a TSK-GEL G3000PWXL gel permeation chromatography (GPC, TOSOH, Japan). The mobile phase was 2.84% Na_2SO_4 solution, with a flow rate of 0.6 mL/min. The standard samples were Dextran Standards with molecular weight of 8.45×10^5, 2.77×10^5, 1.96×10^5, 1.24×10^5, 4.35×10^4, 2.14×10^4, 9.90×10^3 and 4.40×10^3.

2.4.2. Degree of substitution (DS) of agarose and κ-carrageenan derivatives

2.4.2.1. Elemental analysis. The content of sulfur (S%) was determined on a Vario EL CHNS elemental analyzer (Elementa, Germany). The DS was calculated as follows: $DS = (306 \times S\%)/(32–102 \times S\%)$, where: 32 for the atomic weight of sulfur; 102 for the increment in molecular weight when –OH was replaced by $–OSO_3Na$. The sulfate content (SO_4%) was calculated as 3 times of S%.

2.4.2.2. Chemical analysis (Dodgson & Price, 1962). Phenol–sulfuric acid method was used for the determination of the content of polysaccharide. The equation of standard curve was: $y = 0.00679 + 0.00473x$, $R^2 = 0.9888$, where y for the OD_{490nm}, and x for the concentration of D-galactose solution.

Barium chloride–gelatin method was used for the determination of SO_4%. The equation of standard curve was: $y = 0.00142x - 0.02435$, $R^2 = 0.9878$, where y for the OD_{400nm}, and x for the concentration of sulfate solution. The κ-carrageenan sulfate was hydrolyzed by concentrated HCl before determination. The DS was calculated as follows: $DS = (SO_4\% \times 1.6875)$/polysaccharide content of κ-carrageenan, where: 1.6875 for the ratio of the molecular weight of D-galactose and the molecular weight of sulfated group in κ-carrageenan disaccharide units.

2.4.3. Molecular structure

IR spectra were recorded on an EQUINOX-55 FT-IR spectrometer (Bruker, Germany) using KBr pellet method.

^1H and ^{13}C NMR spectra were collected on an AVANCE AV 500 NMR spectrometer (Bruker, Swiss). 20 mg carrageenan sample was dissolved in 0.5 mL d_6-DMSO, and the final concentration of the solution was 40 mg/mL. Choosing TMS as the standard material, system temperature = 43 °C, ^{13}C resonance frequency = 500MH_z, scanning number of ^{13}C NMR > 60,000, scanning time > 40 h.

2.5. Anticogulant activities of sulfated agarose, carrageenan and its derivatives

2.5.1. Rabbit whole blood method

100 IU/mL heparin sodium standard solution was prepared with sterile water and stored at 4 °C before use. And then, the heparin sodium standard solution was accurately measured and mixed with 0.9% sodium chloride solution to form dilute solutions of 40, 30, and 20 IU/mL. The clotting time of the three different concentrations of heparin sodium standard solution were determined, and the standard curve was obtained when the clotting time was plotted against the anticoagulant activity of heparin sodium. The linear equation was $y = 3.155x - 33.75$, where y for the clotting time, and x for the anticoagulant activity (IU/mL).

The polysaccharide samples were dissolved in 0.9% saline solution (0.3% for agarose, sulfated agarose, κ-carrageenan, κ-carrageenan oligosaccharide, and desulfated κ-carrageenan; 0.15% for sulfated κ-carrageenan, ι-carrageenan, and λ-carrageenan). Then 0.1 mL carrageenan solution was added into a blood collection tube without anticoagulant (Guangzhou Improve Medical Ltd., China), using the three different concentrations of heparin sodium standard solution as positive control, and the saline solution as negative control. After all the sample and control solutions were added, the tubes were evacuated by vacuum-pumping. 1.0 ± 0.1 mL rabbit blood was injected into each vacuum test tube followed by gentle shaking, and then quickly put in a water bath of 37 ± 0.5 °C and started timing. The whole process was controlled to be less than 3 min. The equivalent heparin potency of each anticoagulant substance could be found on the standard curve according to its clotting time, and the real potency of anticoagulant substance was the ratio of the equivalent heparin potency to the concentration of the anticoagulant substance. The differences between experimental groups were compared by ANOVA (analysis of variance) using the SPSS software ($p < 0.05$).

2.5.2. APTT and PT test

The activated partial thromboplastin time (APTT) test and the plasma prothrombin time (PT) test were used for the determination of anticoagulant activity in vitro. The procedure of APTT test was as follows: fresh human blood was extracted into vacuum blood collection tubes (sodium citrate 1:9, from Guangzhou Improve Medical Ltd, China) by constantly shaking and mixing, and then rapidly centrifuged (3000 rpm) for 10 min to isolate the plasma for use. The polysaccharide samples were dissolved in saline solution (0.9%) to form 0.003% (for sulfated agarose, sulfated κ-carrageenan, ι-carrageenan, and λ-carrageenan) or 0.3% (for κ-carrageenan) solution. 125 μL carrageenan sample solution was taken into each plastic test tube, and then 250 μL human plasma was added. After mixing, 0.1 mL plasma mixture and 0.1 mL kaolin partial thromboplastic reagent were added by an CA-1500 automatic coagulation analyzer (Sysmex, Japan), and incubated at 37 °C for 3 min, and then 0.1 mL incubated calcium chloride (0.025 mol/L) was added into the mixture, then automatically recorded the clotting time of the plasma. The procedure of the PT test was the same as that of the APTT test before incubation, except that the time for incubation was 30 s, and the volume of 0.025 mol/L calcium chloride used was 0.2 mL.

2.6. Cytotoxicity of sulfated agarose, carrageenan and their derivatives

The cytotoxicity of sulfated agarose, carrageenan and their derivatives was determined by MTT assay using human umbilical vein endothelial cells (HUVEC). After the monolayer of HUVEC (5000 cells per well) was formed, 100 μL polysaccharide solution with different concentrations (1000, 800, 600, 400, 200, 100, 50, 20, 10, and 5 μg/mL) prepared by 1640 medium (containing 10% FBS and 0.1% double resistant) was added into the 96-well plate. The cells were cultured at 37 °C for 48 h, each concentration having 5 duplications and using culture medium as control. The OD value of the resulting system was measured at 570 and 630 nm using a 680 ELISA detector (Bio-rad, USA). The differences between experimental groups were compared by ANOVA using the SPSS software ($p < 0.05$).

3. Results and discussion

3.1. Purification of κ-carrageenan

Generally commercial κ-carrageenan is a mixture of different types of carrageenans (such as ι-, λ-, β-, etc.), containing a certain amount of water-soluble non-carbohydrates and metal ions. Therefore, it is necessary to purify the raw κ-carrageenan before use. The S% of the κ-carrageenan before and after purification is 4.40 and 6.95 (see Supplementary Table 1), which proves that the impurities has been effectively removed by the KCl precipitation method. The C, N, and S% of the purified κ-carrageenan are close to the commercial sample from Sigma. Compared with the theoretical data, the higher C/S ratio of the purified sample might be due to the existence of some disaccharide units without sulfated groups, and the relatively lower C and S% values and higher H% value might be owing to the adsorptive water after the freeze drying.

3.2. Characterization of agarose, carrageenan and their derivatives

3.2.1. IR analysis

The FT-IR spectra of agarose, carrageenan, and their derivatives are shown in Fig. 2. The main adsorption peaks can be assigned to (Chopin & Whalen, 1993): O—H, near 3400 cm^{-1}; C—H of sugar rings, at 2920 cm^{-1}; C—O of adsorptive water, near 1640 cm^{-1} (Fig. 2a); S=O of sulphate esters, near 1250 and 1370 cm^{-1}; C—O—C of sugar and C—O—S of sulphate esters, at 950–1100 cm^{-1}; C—O of 3,6-anhydro-D-galactose, at 930 cm^{-1} (Fig. 2c); C—O—S of galactose-4-sulphate (G4S), at 845 cm^{-1}; C—O—S of galactose-2-sulphate (G2S) and galactose-6-sulphate (G6S) in λ-carrageenan, at 830 and 820 cm^{-1}; C—O—S of 3,6-anhydrogalactose-2-sulphate (A2S), at 805 cm^{-1} (Fig. 2g); and C—O—S of galactose-6-sulphate (G6S), at 810–815 cm^{-1}. The peaks at 930 and 2920 cm^{-1} of sulfated agarose (Fig. 2b) and sulfated κ-carrageenan (Fig. 2d) still exist, indicating that both the 3,6-anhydro bond and the sugar ring keep intact after sulfation. Only when the chlorosulfonic acid was excessively used, do the absorption peaks of the product at 845 and 930 cm^{-1} disappear, and the absorption peak at 620 cm^{-1} which stands for the S=O bond becomes stronger (Supplementary Fig. 1g), which indicates the rupture of the glycosidic bond. Chemical analysis (Supplementary Table 2) also shows abnormal high DS of the product, suggesting the decrease in sugar content. Both of the sulfated agarose and κ-carrageenan have the strongest peak at 1228 cm^{-1}, which stands for the total sulfate content, indicating that they have the maximum DS. And they also have a strong absorption peak at 812 cm^{-1}, which implies that the replacement largely takes place on G-6. The broad width of the peak also declares that overlapped peaks for other substitution positions may exist. As for the desulfated κ-carrageenan (Fig. 2f), there are still weak absorption peaks near 845 and 1250 cm^{-1}, indicating that the sulfated group does not be completely removed, and the peaks near 930 cm^{-1} proves that the sugar ring structure does not be destroyed during the desulfation process. There are adsorption peaks at 930 and 840–820 cm^{-1} and no peaks at 805 cm^{-1} for λ-carrageenan (Fig. 2h), illustrating the existence of 3, 6-anhydro bond, G2S and G6S, and the nonexistence of A2S. The relatively weak adsorption at 1280 cm^{-1} also indicates the lower DS of the sample.

3.2.2. Molecular weight and sulfate content

The molecular weight and SO_4% of agarose, carrageenan and their derivatives are shown in Table 1. The molecular weight of commercial carrageenans changes in the range of $4\sim10 \times 10^5$, increasing from κ- to ι- and λ-, while their sulfate contents are much lower than the ideal ones (κ-carrageenan, 20.85%; ι-carrageenan, 28.5%). The λ-carrageenan gives the lowest value, 18.51%, even lower than that of pure κ-carrageenan.

After degraded for 46 h, the M_w of carrageenan decreases dramatically to 1.7×10^4 from 3.8×10^5, accompanied with a decrease in PDI from 3.7 to 1.8. The extension of the degradation time to 66 h causes a sharp drop in the intrinsic viscosity of κ-carrageenan, from 303.4 mL/g to 5.68 mL/g. The κ-carrageenan oligosaccharide with M_w of 3.4×10^3 and PDI of 1.3 can also be obtained with intact chemical structure (Fig. 2e) but a little decrease in DS, from 0.85 to 0.74.

From Table 1 we can see that, the SO_4% of both sulfated agarose and carrageenan are much greater than that of raw materials, and are oversulfated. Under the same sulfation conditions (pyridine/chlorosulfonic acid = 5:1 (v/v), 65 °C, 4 h), the DS of the sulfated agarose can be increased by 1.8, but for carrageenan, only by 1.2. Meanwhile, the DS of sulfated κ-carrageenan prepared with different designed sulfation conditions can only be increased by 0.7–1.7 (Supplementary Table 2). It might be explained that the negative charges of the sulfate groups hinder the sulfation reagent from attacking and activating the hydroxyl groups on the sugar ring in the nucleophilic substitution. It was reported that selective sulfation of carrageenan could be achieved under or below room temperature (Araújo et al., 2013). If the temperature increases, the total sulfation reaction will be accomplished, as described here. Besides that, another important factor is the amount of chlorosulfonic acid, which will influence the molecular weight of the products. When the volume ratio of pyridine to chlorosulfonic acid reaches to 5:3, carrageenan is degraded seriously, with the destruction of sugar ring and the cleavage of glycosidic bonds (Supplementary Table 2). The reaction time seems to have the minimal influence on the sulfate content of the products, indicating that the sulfation rate is rather fast. With the extension of the reaction time, the degree of the sulfate substitution increases. But if the reaction time is more than 6 h, the substitution degree decreases instead, which indicates an increase in side reactions. The thorough sulfation can also lead to a large decrease in molecular weight, by almost 96%.

As for the desulfated carrageenan, the extension of the reaction time will not influence the desulfuration degree. The SO_4% values are both reduced to one third of the original κ-carrageenan after either 4h's or 24h's reaction. The shorter time is preferred since longer time will result in more decreases in molecular weight.

3.2.3. NMR

The ^{13}C and ^{1}H NMR spectra of agarose, carrageenan and their sulfate derivatives are shown in Figs. 3 and 4. The corresponding data are shown in Supplementary Tables 3 and 4. The ^{13}C NMR spectrum of agarose or carrageenan (Fig. 3a and c) consists of 12 signals (despite some overlapped signals: A-2 and G-2 overlapped in agarose, A-3 and G-3 overlapped in carrageenan), assigned to 12 carbon atoms with various chemical environments (Goncalves et al., 2005; Rochas & Lahaye, 1989; Yu et al., 2002). However, the ^{13}C NMR spectra of the totally sulfated polysaccharides become rather complicated. This is probably due to the conformational changes of sugar rings caused by substitutions at different carbons and the degradation of polymeric chains.

In the ^{13}C NMR spectrum of the sulfated agarose (Fig. 3b), most carbons generate multiple peaks, showing that the product is a blend of polysaccharides with different configurations (or conformations) and molecular weights. The G-3 signal at 80.3 ppm becomes two groups around 81 ($G_{3\alpha}$) and 83 ppm ($G_{3\beta}$), which only appear in degraded agarose (Lahaye & Yaphe, 1989), manifesting the degradation of the polysaccharide. The facts that the G-6 signals at 58.9 and 60.7 ppm (assigned to G-6 in G-DA2S and G-DA unit, respectively) largely shift downfield to 64.9 and 65.5 ppm (α-shift, assigned to G-6 in G6S-DA and G6S-DA2S unit, respectively), and

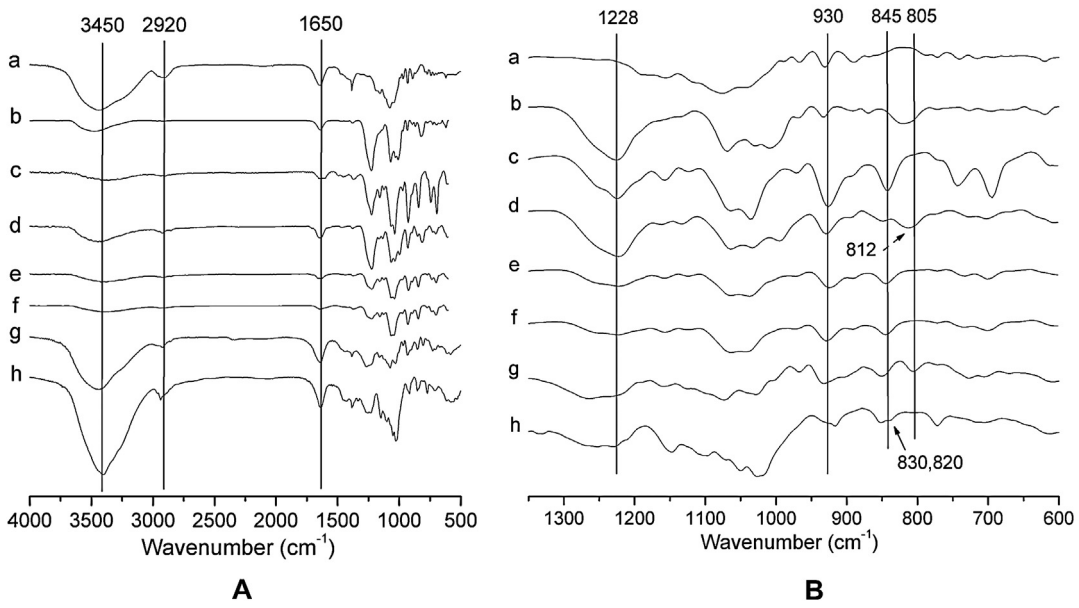

Fig. 2. FT-IR spectra of agarose, carrageenan and their derivatives A: full spectrum; B: partial spectrum; (a) agarose (b) sulfated agarose (c) κ-carrageenan (d) sulfated κ-carrageenan (e) κ-carrageenan oligosaccharide (f) desulfated κ-carrageenan (g) ι-carrageenan and (h) λ-carrageenan.

the G-5 signals (containing several signals resulted from degradation and conformational change) shift upfield from around 76 ppm to around 73 ppm (β-shift), manifest the major substitution on G-6. In addition, the A-2 and G-4 signals at 70.0 and 69.3 ppm partly shift downfield to 74.6 and 73.8 ppm respectively as well, implying that partial substitutions also take place on A-2 and G-4. And there are no signal shifts detected for G-2, indicating that G-2 is the least active carbon during sulfation. Meanwhile, the signals at 3.65, 3.92 and 3.96, 4.38, 5.12 and 5.18 ppm (assigned to G-5, G-6b and G-6a in G6S unit, A-2 in DA2S unit, A-1 in DA2S unit, and A-1 in G6S unit) in ^{1}H NMR spectrum (Fig. 4b) of the sulfated agarose also give evidences of the G-6 and A-2 substitution. The indistinctive decrease in signal intensity at 3.38 and 3.80 ppm (assigned to G-6b in G unit and A-2 in DA unit) and the weak intensity at 3.92, 3.96, 4.38, 5.12 and 5.18 ppm indicate that the substitution is incomplete. No evidence can be given for G-4 and G-2 substitution from ^{1}H NMR spectrum. Compared with the strength of the signals before and after sulfation, the reactivity order of the carbon atom in agarose disaccharide unit could be decided as: G-6, A-2 > G-4 > G-2.

The ^{13}C NMR spectrum of the sulfated carrageenan (Fig. 3d) under the same reaction conditions is simpler than that of the sulfated agarose, illustrating the degradation degree is lower. Similarly, the nearly complete substitution of G-6 can be proved by the great loss of the signals at 59.9 and 75.0 ppm, and the appearance of the signals at 66.6 and 66.8 ppm (assigned respectively to G-6 in G4S6S-DA and G4S6S-DA2S/G2S4S6S-DA2S unit), as well as 72.9 and 73.2 ppm (assigned respectively to G-5 in G2S4S6S-DA2S and G4S6S-DA2S/G4S6S-DA unit). The partial substitution of A-2 is also manifested by the α-shift of the signal from 71.0 ppm to 73.8 and 74.3 ppm (assigned respectively to A-2 in G2S4S6S-DA2S and G4S6S-DA2S unit). There is still no signal shift detected for G-2 in ^{13}C NMR spectrum. However, many evidences have proved that G-2 has also been replaced by $-OSO_3$ group, such as the obvious signals at 95.6 and 100.0 ppm, which are assigned to A-1 and G-1 in G2S4S6S-DA2S unit. The ^{1}H NMR spectrum (Fig. 4d) can further confirm the total sulfation of carrageenan through the signals at 5.25, 5.15 and 5.08 ppm, which is assigned to A-1 in G2S4S6S-DA2S, DA2S, and G4S6S unit, respectively. From the peak area, it is clear

Table 1
Viscosity, molecular weight, and sulfate content of agarose, carrageenan and their derivatives.

Sample	η (mL/g)	M_v ($\times 10^4$)	Mw ($\times 10^4$)	PDI	SO_4%	DS
Agarose	242.5	17	–	–	–	–
Sulfated agarose[a]	–	–	0.68	1.79	35.37	1.81
κ-Carrageenan	303.4	35	38	3.7	20.85	0.85
Degraded κ-carrageenan[b]	80.6	7.6	–	–	–	–
Degraded κ-carrageenan[c]	18.5	1.4	1.7	1.8	–	–
Degraded κ-carrageenan[d]	5.68	0.37	–	–	–	–
κ-Carrageenan oligosaccharide	–	–	0.34	1.3	18.63	0.74
Sulfated κ-carrageenan[a]	–	–	–	–	38.55	2.08
Desulfated κ-carrageenan[e]	–	–	–	–	6.63	0.22
ι-Carrageenan	–	–	45–65[f]	2.0–2.3[f]	28.5	1.30
λ-Carrageenan (low DS)	–	–	99[f]	2.2[f]	18.51	0.73

[a] Sulfation temperature 65 °C, sulfation time 4 h, pyridine–chlorosulfonic acid = 5:1 (v/v).
[b] Degradation time 20 h.
[c] Degradation time 46 h.
[d] Degradation time 66 h.
[e] Desulfation temperature 100 °C, desulfation time 24 h, dosage of κ-carrageenan used for one portion = 0.5 g.
[f] From reference Uno et al. (2001).

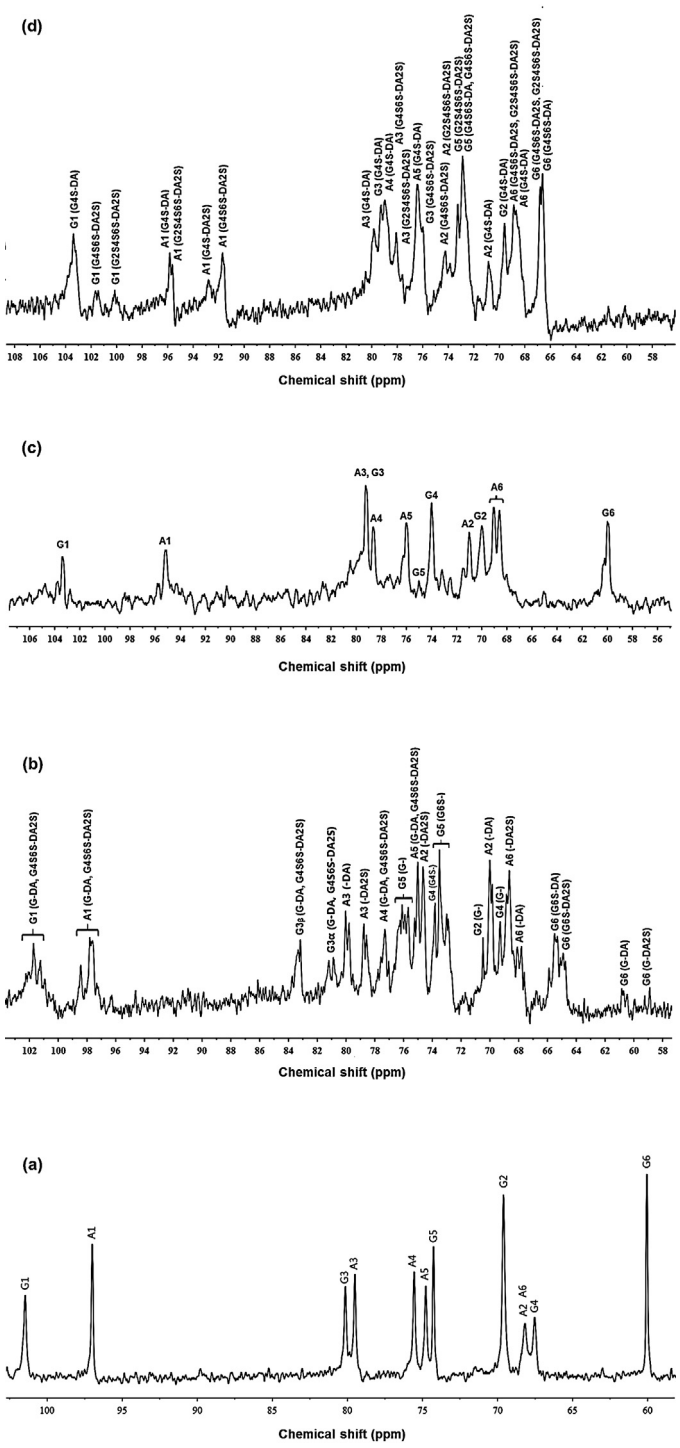

Fig. 3. ^{13}C NMR spectra of agarose, carrageenan and their sulfated derivatives (a) agarose (b) sulfated agarose (c) κ-carrageenan and (d) sulfated κ-carrageenan.

that the totally substituted product is predominant in the polysaccharide blends. The complete disappearance of signals at 3.52 and 3.58 ppm (assigned to G-5 and G-6a in G4S-DA unit), and the α-shift of G-6a to 4.04 ppm, further prove the nearly complete substitution of G-6. Similarly, the α-shift of A-2 (at 4.36 ppm) and G-2 signals (at 3.36 and 4.09 ppm) can also be detected. Therefore, the reactivity order of the carbon atom in carrageenan disaccharide unit could be decided as: G-6 > A-2 > G-2. From the facts that the DS of the carrageenan before sulfation is 0.85, and the DS of the sulfated carrageenan under our sulfation conditions ranges from 1.5–2.5, it

Table 2
Anticoagulant potency of carrageenan and its derivatives by rabbit whole blood method, APTT and PT test.

Samples	Anticoagulant potency (IU/mg)	[a]APTT (min)	PT (min)
Rabbit blood	–	–	–
Heparin sodium	200.30 ± 6.99	–	–
λ-Carageenan	12.23 ± 0.66	75.4	15.8
ι-Carageenan	8.89 ± 0.37	86.4	16.1
Sulfated κ-carageenan	9.87 ± 0.63	60.7	–
Sulfated agarose	5.29 ± 0.10	53.8	–
κ-Carageenan	4.13 ± 0.02	42.4	15.4
κ-Carageenan oligosaccharide	0	–	–
Desulfated κ-carageenan	0	–	–
Agarose	0	–	–

[a] The clotting time of plasma in APTT and PT assay was 35.5 min and12.6 min, respectively.

can be easily deduced that the DS of G-6 ranges from 0.65–1. The DS of both the agarose and carrageenan can be increased by the maximum value of 1.8 through the total sulfation method.

3.3. Effects of agarose, carrageenan and their derivatives on anticoagulation

Results of the anticoagulant potency of agarose, carrageenan and their derivatives are shown in Table 2. The low DS λ-carrageenan demonstrates the highest anticoagulant activity in rabbit whole blood test, but merely 6% compared with heparin sodium. The κ-carageenan oligosaccharide, desulfated κ-carageenan and agarose show no anticoagulant activities. For oligosaccharide, it is probably due to the lack of the secondary structure caused by the decrease in molecular weight; for desulfated κ-carageenan and agarose, it might be due to the lack of favorable sulfate groups. Comparing the anticoagulant potencies of carrageenans with the similar DS and the same concentration, such as κ-carageenan (DS = 0.85), κ-carageenan oligosaccharide (DS = 0.74) and λ-carrageenan (DS = 0.73), we can find that the anticoagulant activity is independent of the DS, by showing values of 4.13, 0, and 12.23 IU/mg, respectively. Moreover, both the sulfated agarose (DS = 1.81, M_w = 6.8 KDa) and the sulfated κ-carrageenan (DS = 2.01) have the highest DS, but their anticoagulant potency is merely 5.29 and 9.87 IU/mg, respectively, lower or only a little higher than ι-carrageenan (DS = 1.30, M_w = 450–650 KDa), illustrating that the anticoagulant activity will largely depend on the substitution position, and partly on the molecular weight. Moreover, the anticoagulant activity of sulfated agarose is much higher than the κ-carrageenan oligosaccharide (M_w = 3.4 KDa) despite of their similar molecular weights, also indicates that the influence of the molecular weight is not so significant. The existence of G4S and DA2S seems helpful to increase the anticoagulant activity, whereas G6S is not beneficial. The order of the positional influence on the anticoagulant activity is supposed to be: A-2, G-2 > G-4 > G-6.

APTT and PT test demonstrate the anticoagulant mechanism of the carrageenan series being endogenous, that is, they could not inhibit the release of Factor III when contacting with blood and would not prolong the clotting time in PT test. The results of APTT also manifest the DS independent of the anticoagulant activity. The G6S is the least favorable unit to anticoagulant activity, which is different from the result that the 6-sulfate group was very important in determining anticoagulant activity of (1-3)-linked polysaccharides (Chaidedgumjorn et al., 2002). The order of anticoagulant activity of the carrageenans is almost the same as those in rabbit whole blood test, except for ι-carrageenan versus sulfated κ-carrageenan, and λ-carrageenan versus ι-carrageenan. The result of APTT reveals that ι-carrageenan possesses higher anticoagulant activity than sulfated κ-carrageenan, but actually their clotting time

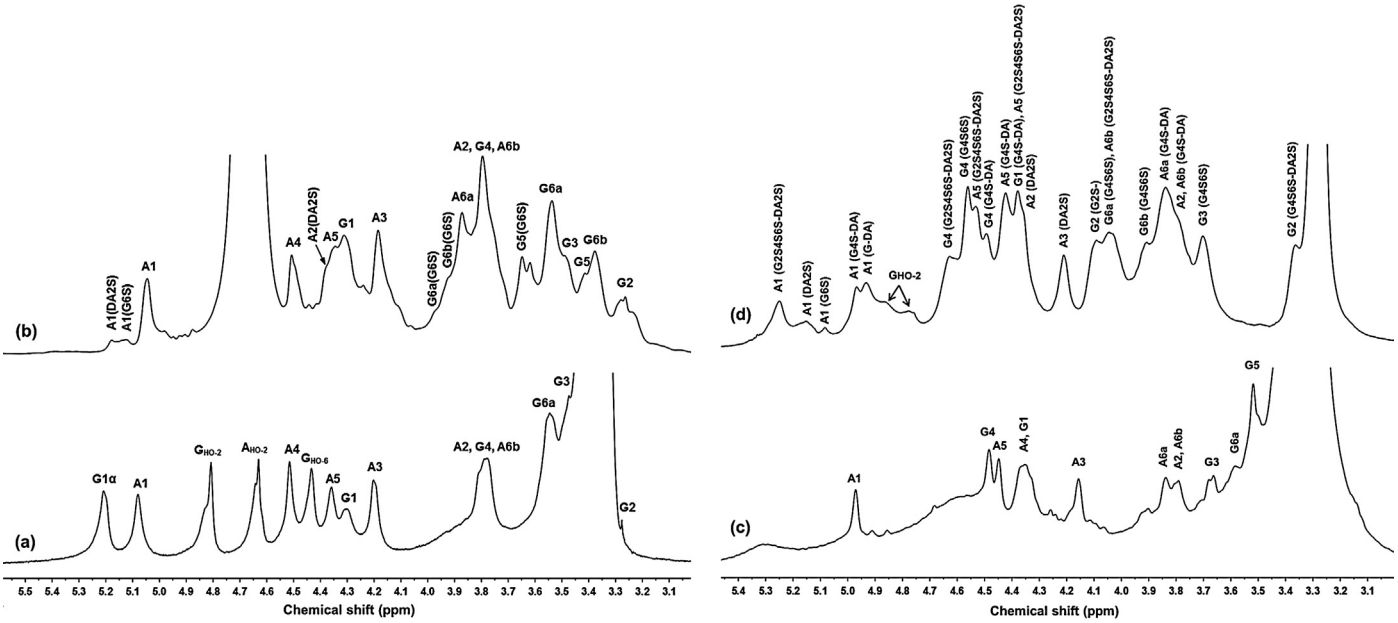

Fig. 4. ^{1}H NMR spectra of agarose, carrageenan and their sulfated derivatives (a) agarose (b) sulfated agarose (c) κ-carrageenan and (d) sulfated κ-carrageenan.

in blood test is similar. The reason is possibly that the media used for the two assays were different, where both plasma and blood cells were contained in the whole blood assay, but only plasma in APTT assay. The result of APTT test is more precise and credible than that of blood test, since the concentration of the drugs is precisely determined. Moreover, in clotting test, platelet would be involved in the clotting process, and the interaction between the drugs and the platelets should be considered. λ-Carrageenan shows much longer clotting time than ι-carrageenan, but the results become opposite in APTT assay. As for λ-carrageenan, the concentration of the drugs in blood assay was 10 times higher than in APTT test. Therefore, λ-carrageenan might have a stronger interaction with the blood cells, and resulted in prolonged blood coagulation. Considering the primary structure of the glycans, the unit containing C-6 substituted sulfate groups, which displays longer clotting time in blood test, might have stronger interactions with the surface proteins of blood cells.

3.4. Effects of κ-carrageenan and its derivatives on growth inhibition of HUVEC

3.4.1. Effect of molecular weight

The molecular weight of κ-carrageenan will influence the cell viability. The HUVEC proliferation is inhibited by κ-carrageenan with high molecular weight in a concentration dependent manner (Fig. 5a). κ-Carrageenan (DS = 0.85) with concentration lower than 0.1 mg/mL has little cytotoxicity on HUVEC. When the concentration is higher than 0.1 mg/mL, κ-carrageenan can significantly inhibit the cell proliferation, and the viability rate even reduces to 60% at a concentration of 1 mg/mL. On the contrary, κ-carrageenan oligosaccharide (DS = 0.74) has no obvious growth inhibition on HUVEC when the concentration ranges from 5 to 1000 μg/mL (Fig. 5b). The cytotoxicity of κ-carrageenan might come from the formation of double helices, which would bind ions and biomolecules directionally through sulfate groups and hydrogen bonds. Considering that both of them have similar sulfate content and sulfation pattern, the distribution of the sulfated groups in the secondary structure of the polymeric chain and the tendency to conduct coil-double helices transformation might play important roles in the interaction between cell surface and the glycan.

3.4.2. Effect of substitution degree

The amount of sulfated groups also has influence on the cell viability. The desulfated κ-carrageenan (DS = 0.22) shows no inhibitory effect on HUVEC growth (Fig. 5g), same as agarose does (Fig. 5c). Apparently sulfate group can be a source of cytotoxicity for sulfated polysaccharides, and the cytotoxicity might increase with rising sulfate groups. In our experiment, it can be suggested that κ-carrageenan with DS of 0.2 have no cytotoxicity.

3.4.3. Effect of substitution position

Similar to κ-carrageenan, the cell growth inhibition of sulfated κ-carrageenan (DS = 2.1) is also concentration-dependent (Fig. 5d). When the concentration is lower than 100 μg/mL, there is no growth inhibition. With the increase of the concentration, the cell viability decreases significantly. When the concentration is higher than 600 μg/mL, the viability reduces to 50%. Meanwhile, the sulfated agarose (DS = 1.8) displays lower cytotoxicity (Fig. 5e) than sulfated κ-carrageenan does, but higher than agarose. These results suggest that the introduction of sulfated group on G-6 might cause the strongest cytotoxicity. With similar sulfate contents, the higher the content of G6S, the stronger the cytotoxicity will be.

Furthermore, ι-carrageenan (G4S-DA2S) exhibits no cytotoxicity and promotes the proliferation of HUVEC when the concentration ranges from 5 to 1000 μg/mL (Fig. 5f), indicating that the substitution on A-2 may eliminate the cytotoxicity and benefit the cell growth. The low DS λ-carrageenan also shows less cytotoxicity (Fig. 5h) than pure κ-carrageenan (G4S-DA), although pure λ-carrageenan (G2S-A2S6S) has been reported to have a certain inhibition effect on HUVEC growth (Chen, Yan, Li, Wang, & Xu, 2007). From the facts mentioned above, the order of the cytotoxicity of sulfated group in red seaweed galactan is supposed to be: G-6 > G-4 > G-2 > A-2. A synergistic effect of both G4S and DA2S/α-1, 4-G2S is significant to weaken or eliminate the cytotoxicity. It is probably because the existence of DA2S/α-1,

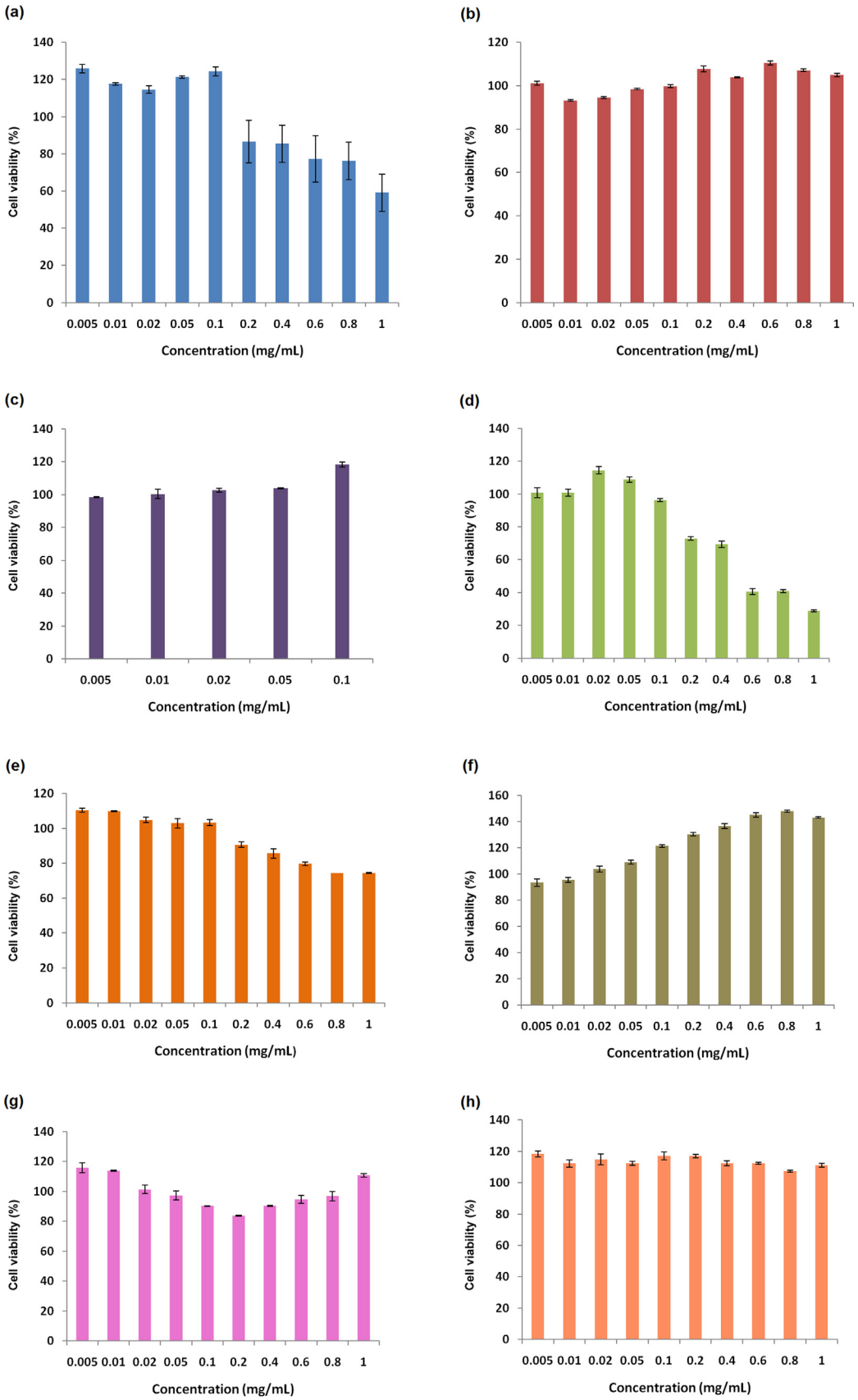

Fig. 5. Effect of red seaweed polysaccharides and their derivatives on HUVEC viability (a) κ-carrageenan (b) κ-carrageenan oligosaccharide (c) agarose (d) sulfated κ-carrageenan (e) sulfated agarose (f) ι-carrageenan (g) desulfated κ-carrageenan and (h) λ-carrageenan.

4-G2S could hinder the formation of the cytotoxic conformation existing in κ-carrageenan, and lead to a change to another non-toxic one. It is shown that at the concentration below 0.1 mg/mL, all the polysaccharides have little cytotoxicity, while above that, different levels of cytotoxic effects are demonstrated, suggesting that the coil-helices transformation begins to form and take effect.

4. Conclusions

Total sulfation of agarose and κ-carrageenan can be achieved by pyridine-chlorosulfonic acid method under higher temperature to increase the DS of polysaccharides by maximum value of 1.8. The reactivity order of the carbon atom in disaccharide unit of agarose is G-6, A-2 > G-4 > G-2, and of carrageenan is G-6 > A-2 > G-2. The anticoagulant activity is largely dependent on the substitution position of the polysaccharides. The existence of G4S and DA2S other than G-6S is helpful to increase the anticoagulant activity. The order of the positional influence on the anticoagulant activity is supposed to be: A-2, G-2 > G-4 > G-6. As for cell proliferation, the introduction of sulfate groups on G-6 will cause the strongest cytotoxicity. The order of the cytotoxicity of sulfate groups is supposed to be: G-6 > G-4 > G-2 > A-2.

Overall, the substitution position rather than the substitution degree of the sulfated red seaweed polysaccharides demonstrates the most significant effect on cell proliferation in our experiment. The secondary structure of the polysaccharides seems to be a key factor to biological activities. The modulation of molecular weights, substitution degree and substitution positions will finally result in the conformation changes. The optimal conformation is supposed to contain a half-rigid $^1C_4$ 3,6-anhydro-link, which is neither apt to bind ions or molecules directionally, as κ-carrageenan does, nor randomly, as λ-carrageenan does.

Acknowledgments

The authors would like to appreciate Dr. Lingmin Zhang for his help in some MTT experiments. This project is supported by the National Science and Technology Support Program (12-5) (2012BAC07B05), the 863 High-Tech Plan (2007AA09Z436), NSFC (31070853), Guangdong Tech Plan (2010B031100015), Guangzhou Tech Plan (2010EJ-0041), Doctoral Fund of Ministry of Education of China (2012), and GPSTD Produce-Learn-Research Guide Project (2011B090400298).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2013.10.010.

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