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Ultrasound-assisted fast preparation of low molecular weight fucosylated chondroitin sulfate with antitumor activity

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ABSTRACT

Fucosylated chondroitin sulfate from sea cucumber *Isostichopus badionotus* (fCS-*Ib*) was depolymerized with an ultrasound-accelerated, metal-free Fenton chemistry, relying on H_2O_2/ascorbic acid redox system. Fragments of different molecular weights were obtained at different reaction temperatures, ascorbic acid concentrations and ultrasonic intensities. The structures of two typical depolymerized fragments were evaluated using high-performance liquid chromatography (HPLC), infrared spectroscopy (IR), and nuclear magnetic resonance spectroscopy (NMR). The results showed that ultrasound enhanced the degradation efficiency of H_2O_2/ascorbic acid system mainly by disaggregating sulfated polysaccharide clusters and that free radicals induced depolymerization with no significant chemical changes in the backbone of fCS-*Ib* and with no obvious loss of fucose branches. The antitumor activity, using A549 lung cancer cells, showed that the ultrasound treated low molecular weight sulfated fragment enhanced proliferation-inhibitory and anti-migration effects, compared to native fCS-*Ib*. This was different from the anticoagulant activity of fCS-*Ib*, suggesting that the molecular weight change may cause a conformational transition and affect biological activity. We propose that combining ultrasound with non-metal Fenton chemistry as an effective method to prepare low molecular weight fCS fragments with potential applications as functional foods, antitumor drugs, and that these fCS fragments display negligible bleeding risk.

1. Introduction

Fucosylated chondroitin sulfate (fCS), a glycosaminoglycan (GAG) obtained from sea cucumbers, is mainly composed of alternating β-D-glucuronic acid and N-acetyl-β-D-galactosamine units (Mourao et al., 1996) with sulfated fucose branches invariably extending from the 3-*O*-position of the β-D-glucuronic acid. This fCS GAG has been developed as a substitute for heparin because of its anticoagulant and antithrombotic activities (Zhao et al., 2015). Recently, fCS also attracted the considerable attention as a potential antitumor therapeutic (He et al., 2014; Zhao, Zhang et al., 2013) and in treating hyperlipidemia (Li, Li, Yan et al., 2017; Wu et al., 2016). Unfortunately, fCSs can also cause undesirable side effects, including the activation of FXII, platelet aggregation (Chen et al., 2013), hypertension and spontaneous bleeding in humans and some animals (Buyue & Sheehan, 2009), limiting their application in medical uses. Low molecular weight fCS has been reported to retain or even show enhanced biological activities compared to native fCS and to exhibit negligible adverse effects (Sheehan & E. N. W., 2006).

The preparation of low-molecular-weight fCS has been mainly accomplished through acidic, oxidative (Li et al., 2016), combined hydrazinolysis and nitrous acid treatment (Yan et al., 2017; Zhao, Lai et al., 2013) and radiation treatment (Wu et al., 2013), and no enzymes are available for the mild depolymerization of the fCS polysaccharides. The fairly drastic conditions for acid-catalyzed hydrolysis can cause partial loss of sulfated fucose branches and their desulfation, significantly impairing the biological activity of the resulting fragments (Buyue & Sheehan, 2009; Mourao et al., 1996). Hydrazine deacetylation and nitrous acid treatment are effective for backbone depolymerization by cleaving the glycosidic bonds at the reducing end of

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galactosamine units with sulfated fucose branch types and sulfate substituents remained (Yan et al., 2017). Radiation treatment induced depolymerization via selective breakage of glucuronic acid units in the fCS backbone with high efficiency. However, both of these methods are difficult to scale-up due to the use of toxic chemicals or radioactive ^{60}Co. In contrast, free radical degradation preferentially attacks the non-sulfated D-glucuronic acid residues in the backbone, which causes the depolymerization of fCS without obvious loss of sulfate and fucose branches (Li et al., 2016; Wu et al., 2012). The Fenton system has been widely used to generate free radicals through the activation of hydrogen peroxide (H_2O_2) by reduced transition metals (e.g., Fe^{2+} and Cu^{2+}) and involves a complex reaction sequence (Bokare & Choi, 2014). There have been a number of studies that have applied Fenton chemistry to prepare low molecular weight sulfated polysaccharides. However, despite the high degradation efficiency and the simplicity, the practical application has been severely restricted by strictly acidic conditions and resulting metal ion precipitation (Bokare & Choi, 2014; Garrido-Ramirez, Theng, & Mora, 2010).

Non-metal Fenton process is emerging as alternative methods for efficient polysaccharide depolymerization. The non-metal Fenton system can operate at near-ambient temperatures in the absence of trace metals and over a broad pH range. Among all the non-metal Fenton system, the H_2O_2/ascorbic acid system is the most eco-friendly and represents a highly efficient way to generate strongly oxidizing radical species (primarily $HO^{\cdot}$). In Fenton processes (Siddique, Farooq, & Price, 2014; Zhang, Fu, & Zhang, 2009), a combination of ultrasound and non-metal Fenton process has been also reported to improve the degradation efficiency during polysaccharide depolymerization (Zhi et al., 2017). Unlike some polysaccharides, which can be degraded by ultrasound alone, fCS shows no obvious depolymerization when treated only with ultrasound. Therefore, it was unclear whether ultrasound could provide synergy with a non-metal Fenton system to accelerate the degradation of fCS.

The present study reports the development of high efficiency ultrasound-accelerated, non-metal Fenton-like chemistry, relying on H_2O_2/ascorbic acid, for depolymerization of highly sulfated fCSs obtained from sea cucumber. The effect of reaction temperature, ascorbic acid concentration, and ultrasound intensities on the molecular weight of resulting fragments were determined, their chemical compositions were characterized using Fourier transform-infrared (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy, with the aim of clarifying the mechanism underlying ultrasound accelerated non-metal Fenton depolymerization. Finally, the in vitro tumor cell growth inhibitory effects and anti-migration activity of native fCS and the resulting fragments were investigated on A549 lung cancer cells.

2. Materials and methods

2.1. Materials

Sea cucumbers *Isostichopus badionotus* (from Western Atlantic Ocean) were purchased from a local market in Qingdao, Shandong, China.

2.2. Depolymerization of fCS-Ib by different reaction systems

Crude sea cucumber polysaccharide was prepared using a previously described method (Chen et al., 2011). Ultrasound treatments were performed according to previous studies (Li et al., 2018). Under the selected conditions, ultrasound/H_2O_2/ascorbic acid (ultrasonic intensity, 424 W/cm^2, the concentration of ascorbic acid, 48 mM and the concentration of H_2O_2, 200 mM), the results were compared with: single ultrasound treatments (424 W/cm^2), ultrasound (ultrasonic intensity, 424 W/cm^2) assisted with H_2O_2 (200 mM), ultrasound (ultrasonic intensity, 424 W/cm^2) assisted with ascorbic acid (48 mM), single H_2O_2 (200 mM), single H_2O_2/ascorbic acid system (the concentration of H_2O_2, 200 mM and the concentration of ascorbic acid, 48 mM). All the tests were performed at the temperature of 25 °C for 90 min.

2.3. Depolymerization of fCS-Ib by ultrasound/H_2O_2/ascorbic acid system

The fCS-*Ib* was depolymerized by ultrasound/H_2O_2/ascorbic acid system according to our previous studies (Li et al., 2018). The effects of the following parameters were investigated: ultrasound intensity (61, 182, 303, 424 and 545 W/cm^2), temperature (0, 15, 25 35 and 45 °C) and ascorbic acid concentration (2.0, 8.0, 16, 32.0 and 48.0 mM). The general depolymerization conditions of all treatments were as follow: reaction time of 90 min, temperature at 25 °C, ascorbic acid concentration of 48 mM, hydrogen peroxide of 200 mM, the ultrasound intensity of 424 W/cm^2. NH_4OH solution (5%) was added to terminate the reaction by adjusting pH to 9.0. The depolymerization conditions for preparation of DfCS1 were as follow: reaction time of 90 min, temperature at 35 °C, ascorbic acid concentration of 48 mM, hydrogen peroxide of 200 mM, the ultrasound intensity of 545 W/cm^2 and the conditions of reaction time of 90 min, temperature at 45 °C, ascorbic acid concentration of 48 mM, hydrogen peroxide of 200 mM, the ultrasound intensity of 545 W/cm^2 were used for preparation DfCS2. The depolymerized products were desalinated by dialysis with a 500 Da cut-off membrane for 72 h, concentrated and subsequently lyophilized.

The Mw of depolymerized fCS-*Ib* were determined by gel permeation chromatography (GPC) according to our previously studies (Yan et al., 2017).

2.4. Chemical composition analysis of depolymerized fCS-Ib fragments

The monosaccharide composition of oligosaccharide fragments was determined by the 1-phenyl-3-methyl-5-pyrazolone (PMP) high performance liquid chromatography (HPLC) method (Wu et al., 2013).

2.5. IR spectral analysis

The FT-IR analysis was applied to obtain IR spectra of the depolymerized fCS-*Ib* samples using a Nicolet Avatar 370 instrument. Samples (~1 mg) were ground together with 200 mg KBr, pressed into pellets for IR scanning from 400 to 4000 cm^{-1} with 32 scans and a 4 cm^{-1} resolution.

2.6. NMR analysis of low-molecular-weight fCS-Ib

For NMR analysis, native fCS-*Ib* and DfCS2 (~5 mg) were evaporated with 550 μL of D_2O (99.96%) twice by vacuum freeze-drying before final dissolution in 550 μL of D_2O (99.96%). The samples were acquired in D_2O with chemical shifts expressed in δ PPM. The spectra of ^1H, ^{13}C NMR, homonuclear $^1H/^1H$ total correlation spectroscopy (TOCSY), correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and nuclear Overhauser effect spectroscopy (NOESY) experiments were conducted by a 600 MHz NMR spectrometer (DD2-600; Agilent Technologies Inc., CA, US) at 25 °C.

2.7. Cell viability assay

The antitumor activity of native fCS-*Ib* and DfCS2 on A549 cells was evaluated using MTT assay (Miao et al., 2013).

2.8. Transwell migration assay

Transwell migration assays were performed by using modified Corning's chamber in 24-well cell culture plate with 8 μm pores. Chambers were washed with PBS for three times. Then the medium with the tested compound was placed in the lower chamber, and cells were seeded in the top chamber. Cells were treated with samples at 37 °C in a humidified atmosphere of 5% CO_2. After incubation for 24 h,

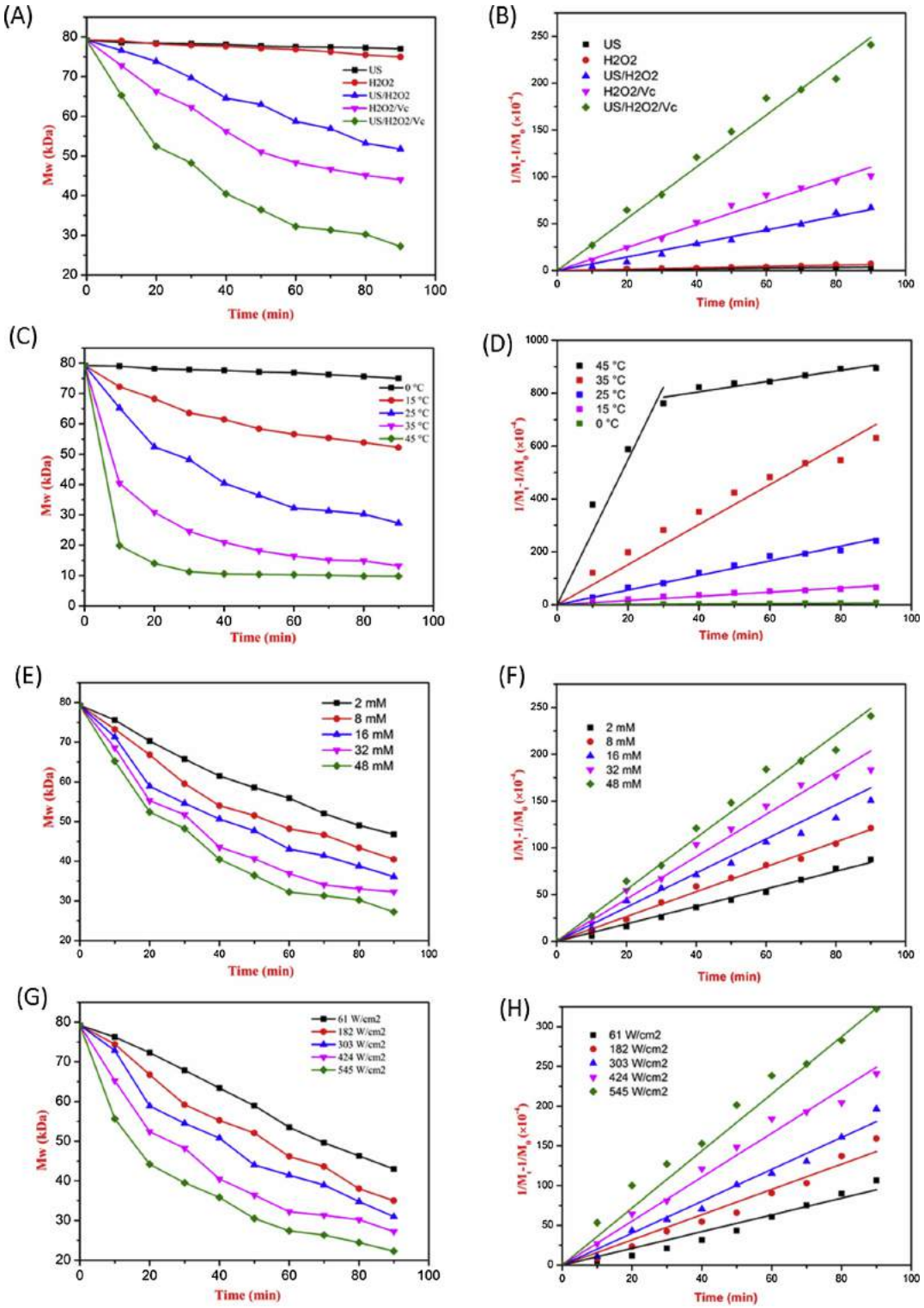

Fig. 1. Effect of different reaction conditions on fCS degradation. (A) Different degradation systems; (B) Kinetics of fCS degradation at different degradation systems; (C) reaction temperature; (D) Kinetics of fCS degradation at different reaction temperatures; (E) Ascorbic acid concentration; (F) Kinetics of fCS degradation at different ascorbic acid concentrations; (G) Ultrasound intensities; (H) Kinetics of fCS degradation at different ultrasound intensities.

non-migrating cells on the top surface of the membrane were gently scraped away with cotton swab. The migrated cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.2% crystal violet. Images were recorded using a Leika inverted microscope.

2.9. Effects of DfCS2 on migration of A549 cells

A549 cells were seeded into a well of 6-well plates (0.5×10^6 cells/well). After 24 h at 37 °C, a wound in the cell monolayer was formed by scraping with a 200-mL tip. After the cells were washed three times with the growth medium, varying concentrations of samples (0, 60, 125, or 250 μg/ml) were added to the wells and incubated for 24 h at 37 °C. Then, migrated cells in the scraped area were photographed under a phase contrast inverted microscope.

3. Results and discussions

3.1. The synergetic effects of sonolysis and H_2O_2/ascorbic acid system to depolymerize fCS-Ib

As illustrated in the Fig. 1, treatment with ultrasound alone led to no obvious reduction of molecular weight. Different from the results obtained for the depolymerization of other polysaccharides, such as pectins and glucans, in which ultrasound alone or with H_2O_2 treatment alone resulted in depolymerization (Guo et al., 2014). Our previous studies demonstrated that ultrasound was an effective method to decrease the molecular weight (Mw) of fucoidan from sea cucumber *Isostichopus badionotus* (Guo et al., 2014). However, even in combination with H_2O_2, the ultrasound failed to degrade the sulfated polysaccharide into low molecular weight fragments within 90 min. This finding suggests that ultrasonic degradation is likely affect the molecular structure and dissolved state of the polysaccharides and the H_2O_2/ascorbic acid system might be a more efficient way to prepare low molecular weight fCS-*Ib* than other ultrasound-based systems.

Next, studies were undertaken to test whether ultrasound-enhanced H_2O_2/ascorbic acid could degrade fCS-*Ib*. Surprisingly, the appearance of 27.2 kDa products (Fig. 1A) and a markedly increased kinetic rate constant k (Fig. 1B) indicated that ultrasound, not only enhanced the depolymerization process but also significantly improved depolymerization efficiency. These results further demonstrated that ultrasound provided synergy with other depolymerization methods such as radical depolymerization (Achour et al., 2013; Petit, Noiret, Guezennec, Gondrexon, & Colliec-Jouault, 2007).

3.2. Effects of reaction parameters on fCS-Ib depolymerization

As shown in the Fig. 1C and Table 1, degradation efficiency increased significantly with increased reaction temperature from 0 °C to 45 °C, consistent with the theory that increased temperatures lead to higher average kinetic energy as a result of more molecular collisions per unit time (Yue et al., 2008). However, higher temperature resulted in a lower ultrasonic degradation efficiency of fucoidan from sea cucumber, as the increased reaction temperature allows cavitation to take place with lower acoustic intensity by rising vapor pressure associated with the heated liquid (Guo et al., 2014; Sivakumar, P. A., & Pandit, 2002), suggesting that the possible degradation mechanism by US/H_2O_2/ascorbic acid acting on the fCS-*Ib* is a combination of mechanical forces and free radical chemical depolymerization and that the free radical degradation predominates. The increased kinetic rate constant k observed with elevated reaction temperature (Fig. 1D) further demonstrates that free radicals mainly contribute to fCS depolymerization. However, the kinetic rate constant k decreased sharply after 30 min of degradation at 45 °C, indicating the reaction is likely to be uncontrollable at high temperature. Therefore, 35 °C was selected as the appropriate reaction temperature for high degradation efficiency and controllability.

Table 1
Monosaccharide composition of native fCS-*Ib* and its depolymerized fragments.

Samples	Monosaccharides (mol%)			Mw (kDa)	Yields (%)
	GlcA	GalNAc	Fuc		
fCS-*Ib*	1.43	1	1.71	78.8	–
DfCS1	1.34	1	1.68	14.2	82.3
DfCS2	1.26	1	1.62	9.38	75.4

Molar ratio is expressed as relative to GalNAc. GlcA: Glucuronic acid; GalNAc: *N*-acetyl-D-galactosamine; Fuc: fucose.

The effect of ascorbic acid concentration on the molecular weight of depolymerized fragments are presented in the Fig. 1E. The molecular weight of fCS-*Ib* prepared exhibited a sharp decrease with increasing ascorbic acid concentration from 2 mM to 48 mM for generation of increasing amount of hydroxyl radicals and the results demonstrate that an increased concentration of reactants can speed up the reaction rate over certain range (Fig. 1F).

Ultrasonic intensity is one of the most important factors for affecting the generation of hydroxyl radicals and cavitation bubbles (Guo et al., 2014; Joseph, Puma, Bono, & Krishnaiah, 2009). The effect of ultrasonic intensity on the molecular weight of degraded fragments is presented in the Fig. 1G. The degradation efficiency was improved when the ultrasonic intensity was elevated (Fig. 1H). Therefore, an ultrasound intensity of 545 W/cm^2 was considered suitable for maximum conversion.

It has been reported that the enhanced degradation efficiency of heparin by the combination of H_2O_2 with ultrasonic waves likely results from the increased amount of free radicals (Achour et al., 2013). In fact, the yields of hydroxyl radicals are mainly associated with the sonication frequency and intensity and the free radical chemical effect is prominent in frequency ranges from 200 to 600 kHz (Koda, Kimura, Kondo, & Mitome, 2003). Therefore, in our research the synergistic effect is mainly attributed to the mechanical effect by the ultrasound. In combination with the fact that ultrasound alone treatment leads to no obvious depolymerization of fCS-*Ib* and apparent degradation of fucoidan, we further concluded that the increased degradation efficiency by ultrasound in US/H_2O_2/ascorbic acid system is not caused by shear forces generated by the relative motion between solvent and polymer chains during bubble collapse. In addition, fCS-*Ib* is primarily random linear chains with a few spherical aggregations and polysaccharides intertwined with one another and formed huge aggregates for their fucose branches, while Fuc-*Ib* showed totally spherical structure in images of AFM (Li, Li, Zhi, Wei et al., 2017), suggesting that the overall conformation of polysaccharides may affect a sulfated polysaccharide's sensitivity to ultrasound.

Thiourea was added to the US/H_2O_2/ascorbic acid system as free radical scavenger to identify whether free radical predominates in the depolymerization process of fCS-*Ib*. As is shown in the Supplementary Fig. 1, the addition of radical scavenger can suppress the depolymerization of native polysaccharides in a concentration-dependent manner. When the concentration of the radical scavenger reached 300 mM, no obvious reduction in molecular weight was observed, indicating that the degradation of fCS-*Ib* was significantly influenced by the radical attack and not mainly caused by shear force generated by the relative motion between solvent and polymer chains during bubble collapse, i.e. mechanical or physical effects. The degradation rate of fCS-*Ib* treated with radical scavenger was much lower than untreated group in the first 20 min. However, the degradation rate of fCS-*Ib* treated with radical scavenger increased significantly from 20 to 50 min, suggesting the possibility that when the molecular weight of depolymerized fragments by free radical depolymerization was decreased to certain degree, the glycan chain becomes more flexible and adopted a random coil conformation, resulting in mechanical scission under ultrasound (Yan, Pei, Ma, & Wang, 2015). When the molecular weight of depolymerized

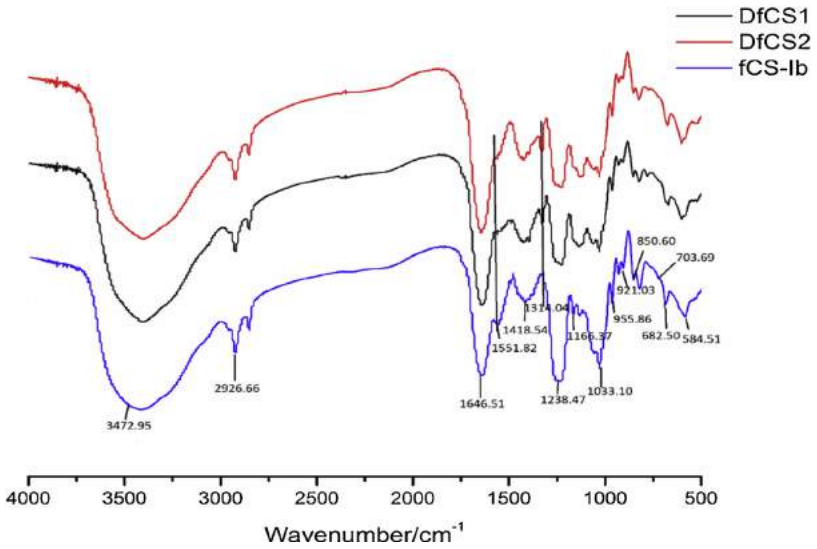

Fig. 2. IR spectra of native fCS-Ib and low molecular weight fCS-Pg fragments prepared by ultrasound/H2O2/ascorbic acid degradation.

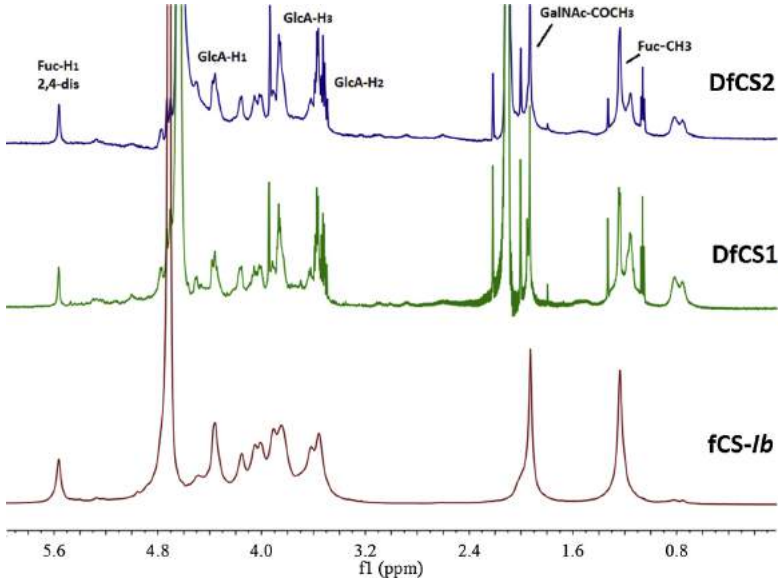

Fig. 3. ^{1}H NMR spectra of native fCS-Ib and depolymerized fragments. DfCS1 and DfCS2 were prepared by US/H_2O_2/ascorbic acid system (ultrasound intensity, 545 W/cm^2; H_2O_2 concentration, 200 mM; ascorbic acid concentration, 48 mM) at 35 °C and 45 °C, respectively.

fragments was further decreased to a limit, the shear force did not work (Gogate & Prajapat, 2015) and the presence of radical scavenger led to reduced degradation.

3.3. Monosaccharide composition analysis

Under optimized conditions (reaction temperature, 35 °C, H_2O_2 concentration, 200 mM; ascorbic acid concentration, 48 mM; ultrasound intensity, 545 W/cm^2) and much severer condition (reaction temperature, 45 °C, H_2O_2 concentration, 200 mM; ascorbic acid concentration, 48 mM; ultrasound intensity, 545 W/cm^2), fCS-*Ib* was cleaved into 14.2 kDa (DfCS1) and 9.38 kDa (DfCS2) (Supplementary Fig. 2), respectively. Chemical compositional analysis of native polysaccharide and the two typical depolymerized fragments were evaluated to obtain primary structure changes information on the depolymerization process. The content of branching was determined to be sulfated α-L-fucopyranose (Fuc)/*N*-acetylgalactosamine (GalNAc) by PMP-HPLC. No significant changes in the molar ratios of GalNAc and Fuc were observed (Table 1). GlcA was gradually reduced at increased reaction temperature, suggesting that GlcA is present at the chain breakage site. In contrast to fCS depolymerization using ^{60}Co irradiation, which causes severe destruction of fCS (Wu et al., 2013), depolymerization using US/H_2O_2/ascorbic acid maintains the chondroitin sulfate backbone in the resulting fragments and depolymerization has limited impact on the fucose branches, critical for the polysaccharide's anticoagulant, anti-metastatic and anti-inflammatory activities.

3.4. IR spectroscopy

The IR spectra were mainly used to determine whether the glycosidic linkage had been altered. The IR spectra of native fCS-*Ib* and its low molecular weight fragments (DfCS1 and DfCS2) are shown in the

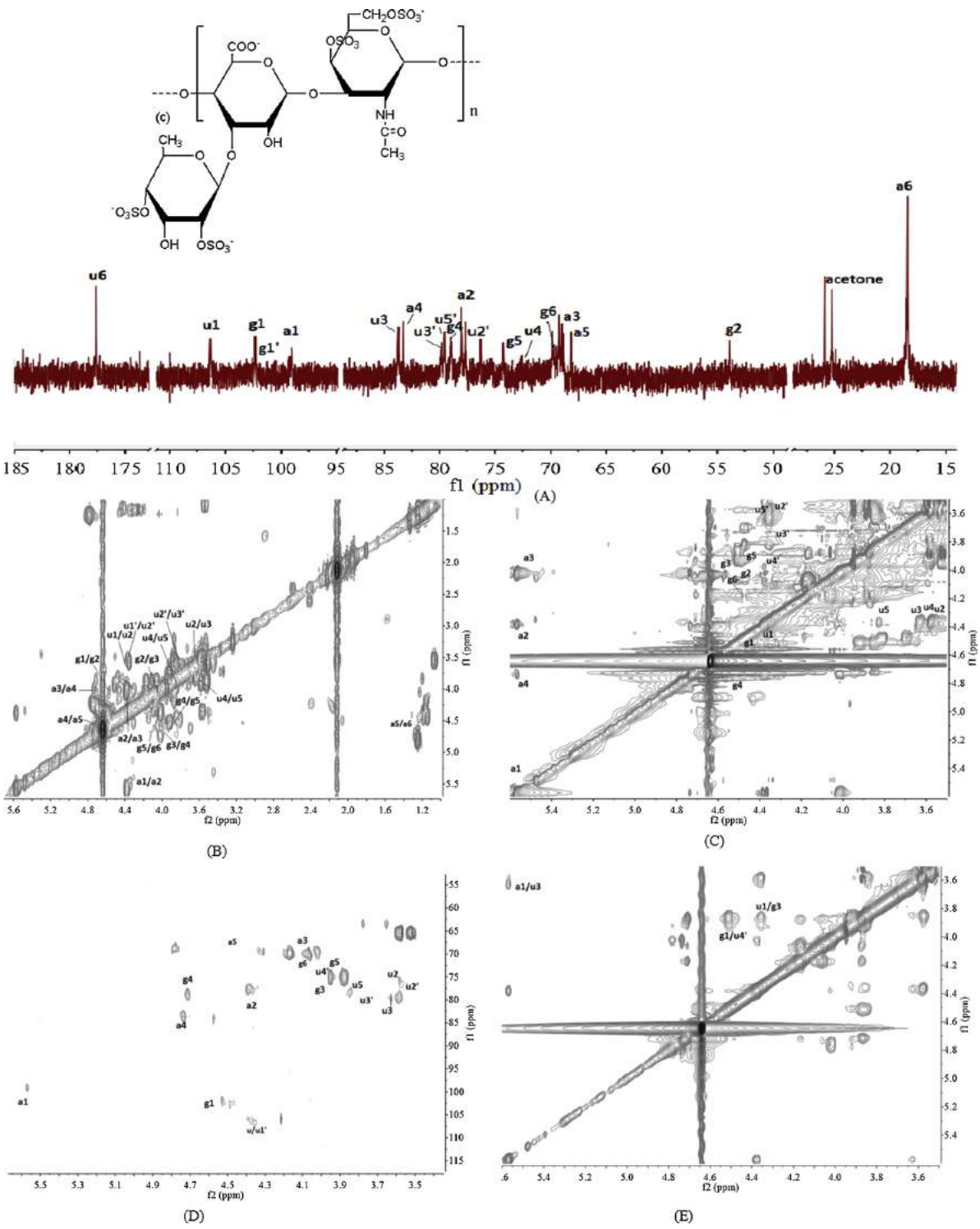

Fig. 4. NMR spectra of DfCS2 (A) ^{13}C NMR of DfCS2; (B) COSY of DfCS2 (C) TOCSY of DfCS2; (D) HSQC of DfCS2; (E) NOESY of DfCS2. Signals designated with a reference to those produced by Fuc2,4S; and signals designated with g, u and u' refer to GalNAc, GlcA and terminal GlcA, respectively.

Fig. 2. Both native polysaccharide and depolymerized fragments exhibit similar bands. In the 4000-1800 cm^{-1} region, characteristic O—H and C—H stretching vibrations at 3472 and 2926 cm^{-1}were observed, respectively. The characteristic bands for GAGs are located at 1800–1400 cm^{-1}, including the amide I band at 1646 cm^{-1}, an amide group II vibration at 1551 cm^{-1} and C—N vibration of the N-acetyl group at 1418 cm^{-1} (Wu et al., 2013). The absorptions at the 820–860 cm^{-1} indicates the pattern of sulfation. The absorption at 825 cm^{-1} suggests the presence of 2,4-O-disulfated fucose or 6-O-sulfated GalNAc and the signal of 850 cm^{-1} corresponds to the presence of

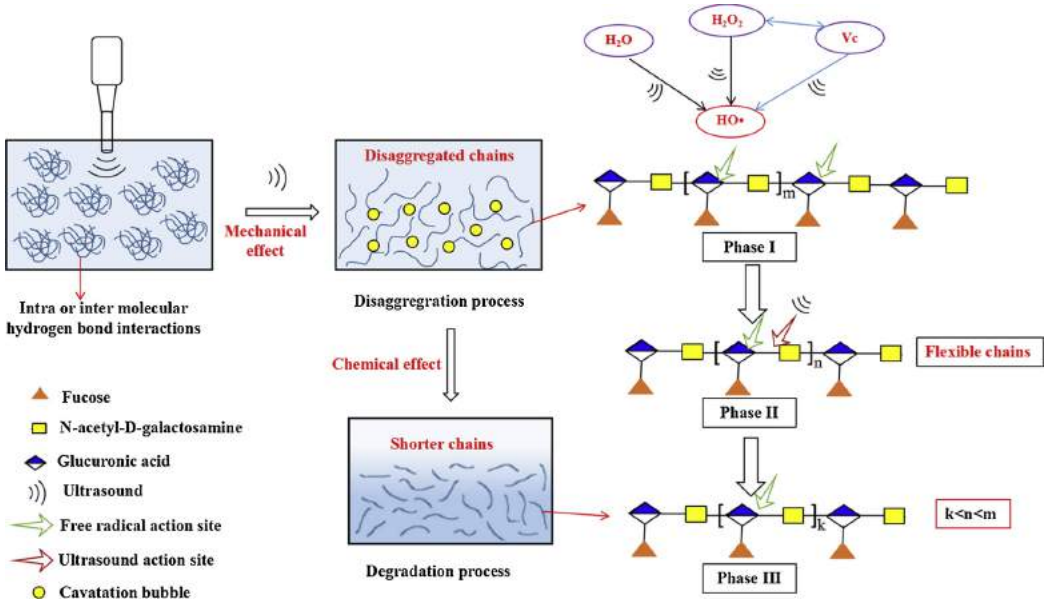

Fig. 5. The schematic diagram of native fCS degradation path by ultrasound/H_2O_2/ascorbic acid system. The ultrasound enhances the efficiency of H_2O_2/ascorbic acid system to degrade fCS through both chemical effects (increasing the amount of hydroxyl radicals) and mechanical effects (disaggregating polysaccharide clusters).

4-O-sulfated Fuc and/or GalNAc (Betty, Igor, O.R., & Rodrigo, 2012), indicating no obvious changes in the sulfation pattern.

However, there are some obvious differences following depolymerization. A decreased signal at 1166 cm^{-1}, assigned to the C—O symmetric stretching vibration of glycosidic linkage, was observed with decreasing molecular weight. The bands at 1033 $cm^{-1}$and 1238 cm^{-1}, attributed to the C—O—C stretching vibration and S═O asymmetric stretching vibration, respectively, were reduced at increased temperatures (Betty et al., 2012; Wu et al., 2013), indicating the importance of reaction temperature under limited conditions.

3.5. NMR analysis

The ^{1}H NMR spectra were investigated to obtain information on structural changes occurring during depolymerization (Fig. 3). Degraded fragments showed similar spectra to native fCS-*Ib* with characteristic signals suggesting that the basic structure of the native polysaccharide was unchanged after depolymerization. Specifically, the peaks at 1.24 ppm correspond to hydrogen atoms in the side-chain sulfated α-L-fucopyranose and the signals at 1.94 ppm result from the methyl protons of GalNAc (CH_3CO) (Wu et al., 2013). The signals between 3.4 and 4.8 ppm are attributable to the cross-ring protons (Mourao et al., 1996; Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992). Based on the previous results (Chen et al., 2011; Li et al., 2016), the major signals at ˜5.54 ppm were assigned to the 2,4-*O*-di-sulfo fucose branches and the unchanged chemical shifts at 2,4-*O*-di-sulfo fucose branches indicate that the depolymerization had no obvious effect on the sulfation patterns of fucose branches.

However, there were some spectral changes following depolymerization. In particular, signals between 3.4–3.8 ppm, assigned to the H-2 and H-3 of GlcA unit, were significantly reduced with decreasing molecular weight, indicating the chain scission by free radical occurred at the GlcA residues, consistent with the finding that GlcA residues of GAGs are very susceptible to free radical attack (Li et al., 2016; Uchiyama, Dobashi) and that the depolymerization of native polysaccharide resulted from the breakage of glycosidic bonds by free radicals, similar to metal-catalyzed Fenton chemistry (Li et al., 2016). These results are in line with the monosaccharide compositional assay.

Limited by the relatively poor resolution of the ^{1}H NMR spectra of the low molecular weight fragments, 2D NMR was employed to further determine the detailed structure of DfCS1 and DfCS2. The assignments of ^{1}H and ^{13}C chemical shifts (Supplementary Table 2) were made from ^{13}C NMR, correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear single quantum coherence (HSQC) spectra and nuclear Overhauser effect spectroscopy (NOESY) spectra (Fig. 4 and Supplementary Fig. 3). The sulfate substitutions were confirmed by comparing the proton chemical shifts of fucose residues in chains to those of the unsubstitituted monosaccharide fucose. The sulfation positions of the Fuc residue were identified as 2,4-*O*-disulfated. The analysis of the COSY and TOCSY (Fig. 4) revealed the β-anomeric configuration of both GlcA and GalNAc. The correlation peaks of g1/u4 (4.52/3.93) and u1/g3 (4.35/3.94) in the NOESY spectrum indicated that GalNAc residue is linked to the C-4 position of the GlcA residues and GlcA residues are linked to the C-3 position of GalNAc residues. Moreover, the correlation peak of a1/u3 (5.54/3.63) confirmed the presence of Fuc residues linked to the GlcA residues. Based on the collective data, DfCS2 was identified as a typical chondroitin E backbone mainly containing 2,4-*O*-disulfated fucose (Fuc2,4S) branches, which was consistent with our previous analysis of fCS-*Ib* (Chen et al., 2011). The results indicate that the ultrasound/H_2O_2/ascorbic acid system cannot only be used for fast preparation of low molecular weight sulfated polysaccharides, but also useful in unraveling the structure of unknown marine GAGs.

Combining the collective data with related literature reports (Achour et al., 2013; Gronroos et al., 2001), a mechanism for ultrasound enhanced H_2O_2/ascorbic acid degradation of fCS-*Ib* can be proposed. Ultrasound waves cause ruptures in the liquid in a form of small bubbles (cavities) filled with vapor and the formation and subsequent collapse of the cavities result in high pressure gradients and high local velocities of liquid layers in their vicinity, inducing shear forces in turn. The shear force produced from cavitation bubbles lower the noncovalent intra-molecular or inter-molecular hydrogen bonds of sulfated polysaccharide chains, causing the disaggregation of polymer clusters. The effect of single ultrasound treatment on conformation change of native fCS was identified by GPC-MALLs. As is shown in the Supplementary Fig. 4, the conformation plot slope of polysaccharide changes

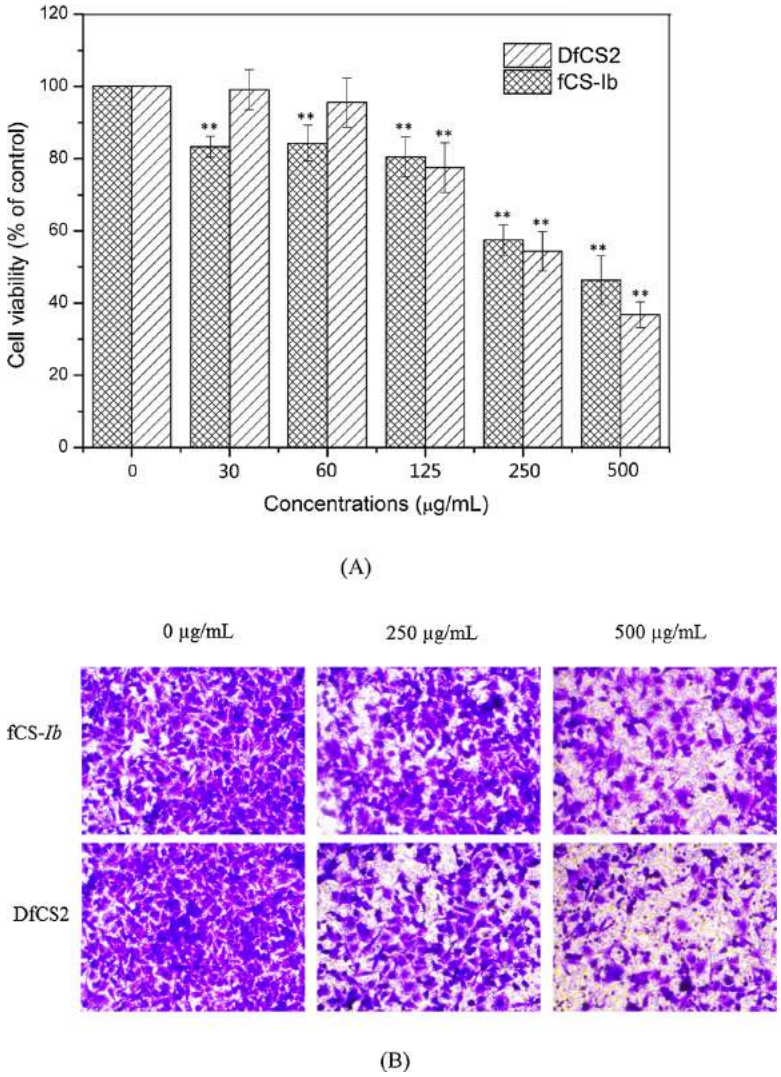

Fig. 6. fCS-*Ib* and DfCS2 inhibited cell viability and cell migration. (A) A549 cells were treated by fCS-*Ib* and DfCS2 for 48 h. Cell viability was measured by MTT assays; (B) Representative images of cell migration assays.

from 0.56 (random coil) to 1.24 (rod-like), suggesting the conformation of the polysaccharide becomes more extended glycan chains in the solution. The sulfated polysaccharide molecules exposed more reactive site in solution for the mechanical action of ultrasound, making these polysaccharide chains more susceptible to free radical attack generated from H_2O_2/ascorbic system (Phase I). This is consistent with the results described in a previous study showing that the shear force generated by the cavitation break up compact random coils (or aggregates) of polysaccharide molecules in solution at the initial of 15 min (Yan et al., 2015). With the decrease in molecular weight caused by free radical degradation, the conformation of the polysaccharide change, becoming more flexible. When sonication time continued, the cleavage of flexible glycan chains taking place by mechanical scission under sonication and free radical depolymerization continued (Phase II) in accordance with the results presented in Supplementary Fig. 1. A characteristic feature of ultrasound degradation process is that there is a definite minimum chain length limiting the degradation process and when it is reached, no further chain scission is observed (Czechowska-Biskup, Rokita, Lotfy, Ulanski, & Rosiak, 2005). When the molecular weight of fCS fragments reached that limit (about 20 kDa), the degradation from shear force disappeared (Phase III). In addition, water is partially dissociated in to OH radicals and H atoms, which makes the sonochemistry of ultrasound similar, in a sense, to radiation chemistry, where at first solvent radicals are formed and subsequently attack the solute (Czechowska-Biskup et al., 2005). These radicals diffuse out of the bubbles and into the solution, leading to the higher concentration of hydroxyl radicals. The backbone of sugar chains is broken by cleaving the glycosidic linkage and the GlcA residues are more susceptible to the OH attack and the more extended and flexible conformation of shorter molecule chains are more reactive with these hydroxyl radicals, thus degrading the sulfated polysaccharide with high efficiency (Fig. 5).

3.6. Cytotoxicity and anti-metastatic activity of native fCS-Ib and DfCS2

As shown in the Fig. 6A, both fCS-*Ib* and DfCS2 inhibit the viability of A549 cells, compared with control cells, in a dose-dependent fashion. Interestingly, fCS-*Ib* exhibited a little higher inhibitory effect than did DfCS2 at relatively low treatment concentrations. While DfCS2 showed better anti-proliferative effect at higher concentrations. The enhanced antitumor activity of DfCS2, when compared with native fCS-*Ib*, may

result from the increased molar concentration resulting from depolymerization. In addition, depolymerization leads to a significant decrease in the molecular weight, thus, causing molecular weight-induced conformational transition and molecular weight-induced conformational transition has been reported in many polysaccharides (Tsaih & Chen, 1997). Therefore, this suggests that depolymerized fCS should have greater tendency to become extended due to the differences in intra-molecular hydrogen bonds and/or charge distribution between smaller and native fCS and were more stiff than native fCS, consistent with a previous study by Wang & Zhang that polysaccharides with higher chain stiffness possessing higher antitumor activities (Wang & Zhang, 2009). Moreover, the extended conformation of low molecular weight fCS might result in more active sites, contributing to the antitumor activity.

Metastasis is the main factor affecting mortality in cancer patients and migration is a critical step during metastasis. Therefore, transwell migration assays were conducted to determine whether fCS-*Ib* and DfCS2 could prevent the migration of A549 cells. As illustrated in the Fig. 6B, the presence of both fCS-*Ib* and DfCS2 significantly attenuated the migration of A549 cells in a dose-dependent manner and DfCS2 showed slightly better anti-migration effect. The effect of native polysaccharide and depolymerized fragment on cell migration was also investigated by wound healing assay (Supplementary Fig. 5) and the migration inhibitory effect of DfCS2 was better than that of native polysaccharide, consistent with the transwell migration assays. Our results from in vitro experiments suggested that low molecular weight fCS-*Ib* was a powerful agent in preventing tumor metastasis, supporting the hypothesis that sulfated polysaccharides may serve as potential anti-metastatic agents. Moreover, the incidence of venous thrombosis is generally high in patients with cancer (Swier & Versteeg, 2017) and inhibition of coagulation also has an effect on cancer patient survival irrespective of the occurrence of VTE (Kakkar et al., 2004). In contrast to sulfated polysaccharides, low molecular weight sulfated polysaccharides possess remarkable anticoagulant activity without bleeding by selectively targeting the intrinsic tenase complex rather than the anticoagulant serpin-related system (Yan et al., 2017), further indicating the significance of low molecular weight sulfated polysaccharides in another potential clinical application.

4. Conclusions

In the present study, an efficient ultrasound enhanced non-metal Fenton redox system relying on H_2O_2/ascorbic acid was developed for fast preparation of low molecular weight fCS from sea cucumbers and the depolymerization mechanism was proposed. The presence of ultrasound can lead to the disaggregation of fCS-*Ib* clusters for its mechanical effects, exposing more acting site attacked by a great amount of hydroxyl radicals generated from ultrasound/H_2O_2/ascorbic acid system. Further structural analysis indicate that the basic structure of sulfated polysaccharide was almost unchanged during the depolymerization process and the free radical preferentially cleaved the GlcA in the backbone, which was similar to other free radical degradation by Fenton systems. The comparison of antitumor activity in vitro using MTT and transwell assays of native polysaccharide and the depolymerized fragment indicates that low molecular weight fCS showed higher antitumor activity against A549 cells. Polysaccharide conformation is associated with the spatial arrangement of the atoms that determine the shape adopted by the chain molecule. A sharp decrease in the molecular weight may result in conformational transition and the polysaccharide conformation change can influence the direct connect between the polysaccharide and the tumor cells. Sulfated oligosaccharide chains with high stiffness may interact selectively with specific sites on the cells, while polysaccharide chains with relatively low stiffness might interact non-selectively with many sites on the cells. The difference in binding affinity of receptors can cause differences in their antitumor activity (Ferreira et al., 2015). However, further studies are required to verify the effect of molecular weight on the conformation of fCS molecules in solution. In conclusion, the present study indicates that US/H_2O_2/ascorbic acid system can be used for fast preparation of low molecular weight sulfated polysaccharides and the low molecular weight sulfated polysaccharide is suitable for use in functional foods and potential therapeutic agents for human cancer.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2018.12.061.

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