**Synthesis of selenium-containing *Artemisia sphaerocephala* polysaccharides:
Solution conformation and anti-tumor activities in vitro**

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Abstract:

It has been reported in our previous work that selenized *Artemisia sphaerocephala* polysaccharides (SeASPs) with the Se content range of 168-1703 $\mu\text{g/g}$ were synthesized by using $\text{Na}_2\text{SeO}_3/\text{HNO}_3/\text{BaCl}_2$ system. In the present work, the solution property of SeASP was studied by using size exclusion chromatography combined with multi angle laser light scattering (SEC-MALLS). A decrease in d_f values indicated that SeASPs with different conformational features that were highly dependent on M_w . SeASPs exhibited a more rigid conformation (d_f value of 1.29-1.52) in low molecular weight range (M_w of $1.026\text{-}1.426\times 10^4$ g/mol) and compact spherical conformation in high molecular weight range (M_w of $2.268\text{-}4.363\times 10^4$ g/mol). It could be due to the degradation of polysaccharide chains in HNO_3 , which was supported in monosaccharide composition analysis. Congo red (CR) spectrophotometric method and atomic force microscopy (AFM) results also confirmed the conformational transition and the evidence on the shape of the rigid chains. In vitro anti-tumor assays, SeASP₂ displayed greater anti-proliferative effects against three tumor cell lines (hepatocellular carcinoma HepG-2 cells, lung adenocarcinoma A549 cells and cervical squamous carcinoma Hela cells) in a dose-dependent manner. This suggested that selenylation could significantly enhance the anti-tumor activities of polysaccharide derivatives in vitro.

Keywords: *Artemisia sphaerocephala*; selenized polysaccharide; solution conformation;

anti-tumor activity

1. Introduction

The biochemical role of selenium (Se) was clearly established almost subsequently with the discovery that the antioxidant enzyme glutathione peroxidase contains a selenocysteine residue at the active site. The upper limit of selenium intake no less than 400 µg/day is prescribed by the Institute of Medicine of The National Academy of Sciences (United States). Selenium deficiency leads to the development of many diseases, such as immune dysfunction and hypothyroidism (Thomas, 2012). Inorganic and organic selenium are the main existence forms of Se in nature. It is believed that the decoration of inorganic selenium with polysaccharides showed higher bioavailability and provided safe and efficient delivery of microelements for humans (Yang et al., 2012; Bondarenko et al., 2016). Selenium-enriched food supplements are the major source of Se, which are generally considered as a dietary supplement (Wang et al., 2013). In organism, the biosynthesis of selenocysteine is very complex due to the inhibition of selenocysteine incorporation and a dramatic alteration in the enzymatic behavior (Thomas, 2012). Therefore, biotransformation and chemical synthesis of selenium containing proteins and polysaccharides have been found to be very useful and attractive.

Selenium-polysaccharides, including functionalized selenium with polysaccharides, natural Se polysaccharides extracted from plants, bio-enrichment method or their chemically synthesized derivatives, are drawing the attention of researchers recently. The bio-activities of Se-polysaccharides are involved in cancer-therapeutic benefit in vitro/vivo (Wang et al., 2013; Shang et al., 2009; Yang

et al., 2012; Wu et al., 2013; Chen et al., 2008), immunoregulation (Mao et al., 2016; Liu et al., 2015; Gao et al., 2016), anti-fatigue (Chi et al., 2015), antibacterial (Lü, Gao, Shan & Lin, 2014), antioxidant and hypolipidemic activity (Mao et al., 2014; Liu et al., 2013). However, the selenium content of natural Se-polysaccharides is very low even in Se-enriched area. Therefore, there has been growing interests in artificial synthetic selenizing polysaccharides due to the efficient and high Se content. Several researchers have shown their results in chemically selenized polysaccharides with different Se contents, i.e. *Radix hedysari* polysaccharide of 3.29 mg/g (Wei et al., 2015), *Atractylodes macrocephala* polysaccharide of 11.27 mg/g (Liu et al., 2015), *Capparis spionosa* polysaccharide of 5.547 mg/g (Ji et al., 2012), garlic polysaccharide of 29.4 mg/g (Gao et al., 2016) and pectic polysaccharide of 478.17 µg/g (Chen et al., 2014b). The selenylation of polysaccharide is one of the most intriguing scientific endeavors in carbohydrate chemistry.

In our previous study, selenized *Artemisia sphaerocephala* polysaccharide (SeASP) with the maximum Se content of 1703 µg/g was synthesized by employing H₂SeO₃ in HNO₃ solution and Ba²⁺ as catalyst. The effect of reaction conditions on the Se contents and yields of SeASPs was studied. The C-6 substituted pattern and a decrease in *M_w* were determined by using ¹³C NMR spectroscopy and size-exclusion chromatograph combined with multi-angle laser light scattering photometer (SEC-MALLS). Compared to the native ASP, SeASP showed greater scavenge hydroxyl and superoxide radical activities in vitro (Wang et al., 2012). Moreover, some researchers have focused on the relations between conformation and biological

activity of modified polysaccharides. The bio-activities and solution conformation are mainly influenced by molecular weight and electrostatic interactions of polysaccharide chains in modified polysaccharides (Tao, Zhang & Peter, 2006; Wang et al., 2016).

The objective of the current study is to investigate the mechanisms governing the relations between chain conformation and structure features of SeASP. SEC-MALLS, monosaccharide components analysis (GC-MS method), Congo red (CR) spectrophotometric method and atomic force microscopy (AFM) were used to determine the chain conformation in solution and molecular topography. Moreover, the tumoricidal effects of ASP and SeASP were evaluated on human hepatocellular carcinoma HepG-2 cells, lung adenocarcinoma A549 cells and cervical squamous carcinoma Hela cells in vitro.

2. Materials and methods

2.1 Materials

Trifluoroacetic acid (TFA), ammonium hydrochloride, pyridine, acetic anhydride and congo red (CR) were purchased from Jingchun Industry Co. Ltd. (Shanghai, China). The standard monosaccharides *L*-Rhamnose (*L*-Rha), *L*-Ribose (*L*-Rib), *L*-Arabinose (*L*-Ara), *D*-Xylose (*D*-Xyl), *D*-Lyxose (*D*-Lyx), *D*-Mannose (*D*-Man), *D*-Glucose (*D*-Glc) and *D*-Galactose (*D*-Gal) were purchased from Sigma Chemical Co. (imported from USA). In this study, all chemicals were of analytical reagent or better grade without further purification. The extraction and purification of ASP was

shown in our earlier study (Wang et al., 2009). The protein (coomassie brilliant blue method), carbohydrate (phenol-sulfuric acid method) and uronic acid (sulfuric acid-carbazole method) contents of ASP was 2.4%, 90.2% and 16.3%, respectively.

2.2 Synthesis of selenized ASP

The preparation of selenized ASP (SeASP) and determination of selenium contents were reported in our previous report (Wang et al., 2012). Briefly, purified ASP was added in HNO₃ solutions (the concentration of 0.4-1.2%) with stirring for 10 h. Then, different amounts of H₂SeO₃ and BaCl₂ were added and reacted for 6 h. After the reaction, the mixture was neutralized (pH value was adjusted to 7-8 with 2 mol/L NaOH) and dialyzed (molecular weight cutoff 8-12 kDa) until the solution was colorless when ascorbic acid was added. SeASP was collected by filtration, washed with ethanol and lyophilized.

2.3 Molecular weight and solution conformation measurements

The weight average molecular weight (M_w) of ASP and SeASPs were determined by using size-exclusion chromatograph combined with multi-angle laser photometer and optilab refractometer (SEC-MALLS, wavelength of 690 nm, Wyatt Technology Co., USA). Ultrahydrogel^{TM} column (7.8×300 mm, Waters, USA) was employed in SEC-LLS measurements. ASP and SeASPs were prepared in ultra-pure water at room temperature with desired concentration of 1 mg/mL and filtered through a cellulose filter (0.45 μm). The elution of the sample was performed using ultra-pure

water as mobile phase at a flow rate of 0.5 mL/min. The injection volume was 50 μ L.

The refractive index increment (dn/dc) value was 0.145 mL/g. The M_w and $\langle S^2 \rangle_z$

were calculated using Zimm method. The equation was shown below

$$\frac{KC}{R_\theta} = \frac{1}{M_w} \left(1 + \frac{16\pi^2 \langle S^2 \rangle_z}{3\lambda^2} \cdot \sin^2 \left(\frac{\theta}{2} \right) \right) + 2A_2C \quad (1)$$

where C was the concentration of the samples (mg/mL); A_2 , the second virial

coefficient; K , an optical constant equal to $[4\pi^2 n_0^2 (dn/dc)^2] / (\lambda^4 N_A)$; R_θ was the

Rayleigh ratio; λ , the wavelength of incident light; n_0 was the refractive index of the

solvent; $\langle S^2 \rangle_z$, the mean square radius of gyration; N_A was the Avogadro' number; θ ,

the scattering angle. The Astra software (Wyatt Tech. Corp.) was employed for data

analysis.

2.4 Monosaccharide components analysis

The monosaccharide composition was detected as previously described (Wang et al., 2014). ASP and SeASP (10 mg) were hydrolyzed with 4 M TFA(4 mL) in a test tube at 120°C for 10 h under airtight condition. The residual TFA was removed by evaporation. The acetylation reaction of hydrolyzate was prepared by adding of ammonium hydrochloride (10 mg), pyridine (1 mL), acetic anhydride (1 mL) and reacting at 90°C for 30 min. The standard monosaccharides including *L*-Rha, *L*-Rib, *L*-Ara, *D*-Xyl, *D*-Lyx, *D*-Man, *D*-Glc and *D*-Gal were prepared in the same way. The analysis of acetate derivatives was carried out by GC-MS (Thermo Polaris-Q) with a HP-5 capillary column. The chromatographic conditions were as follows: high-purity nitrogen gas was at a flow rate of 1 mL/min, temperature of injector and ion source

was 250°C. The initial column temperature was held at 120°C and then increased gradually to 250°C at a rate of 5°C/min.

2.5 Congo red binding studies

Congo red (CR) spectrophotometric method has been commonly used to analyze the solution conformation of ionic biopolymer. The procedure of CR-polysaccharide binding experiment was performed according to the method of Pal et al with some modifications (Pal et al., 2016). CR solution (concentration of 91 µmol/L) and SeASP solutions (concentrations of 0.2~2 mg/mL) were prepared in distilled water at room temperature. Then, 2 mL SeASP solutions were mixed with 2 mL CR solution to give polysaccharide-CR complex by stirring at room temperature for 1 h. UV/Vis wavelength sweeps were performed in the range of 400-600 nm at room temperature (UV1100, Labtech Inc., China). Furthermore, NaOH was added to observe the evolution of maximum absorption wavelength ($\lambda_{\max}$). In brief, 2 mL SeASP solution was mixed with 2 mL CR and 1 mL NaOH solution at room temperature for 1 h, to give a final NaOH concentration of 0.2, 0.4, 0.6, 1.6 and 2 mol/L. Meanwhile, mixed solutions without SeASP or NaOH were prepared as the control.

2.6 Molecular morphology observation

Atomic force microscopy (AFM) was employed to observe the molecular morphology of modified polysaccharide. SeASP was dissolved in HPLC grade water and filtered through a 0.45 µm filter. Then, the solution was diluted to the

concentration of 20, 10 and 1 $\mu\text{g/mL}$ with vigorous stirring for 24 h. About two microlitres of the sample solution was deposited onto freshly cleaved mica and air dried for 4-8 h at room temperature. The sample was examined using a Nanoscope IIIa atomic force microscopy (Veeco Instruments, USA) in tapping mode with commercial RTESP tips (Antimony doped Si, Bruker). The AFM image was obtained with the resolution of 256×256 point.

2.7 Anti-tumor activity in vitro

The anti-tumor activities of ASP and SeASP were evaluated against human hepatocellular carcinoma HepG-2 cells, lung adenocarcinoma A549 cells and cervical squamous carcinoma Hela cells in vitro using CCK-8 (cell counting kit) assay. All cells were obtained from Gansu Academy of Medical Sciences (Gansu, China). Culture medium was 90% Dulbecco's modified Eagle medium (DMEM, HyClone, GE Healthcare, USA) supplemented with 10% fetal bovine serum (Clark, Australia) and penicillin-streptomycin (10 units/mL). Cells were incubated in a 5% CO_2 incubator at 37°C .

HepG-2 cells, A549 cells and Hela cells at a density of 5×10^4 cells per well were placed on 96-well plates, respectively. After cell attachment, the wells were washed and incubated in DMEM media for 24 h. Then, cells were incubated with the addition of polysaccharide samples at various concentrations (25, 50, 100, 150 and 200 $\mu\text{g/mL}$). After 48 h, each well was added with 10 μL of CCK-8 and incubated for another 1 h. Control cells were cultured in medium with no addition of samples. Cell viability was

evaluated by the CCK-8 colorimetric procedure at 450 nm by a micro-plate ELISA reader (Deng et al., 2016). The anti-tumor activity of samples was calculated as the formula:

$$\text{Inhibition rate} = (1 - A_{\text{samp.}}) / A_{\text{contr.}} \times 100\% \quad (2)$$

where $A_{\text{samp.}}$ is the absorbance of polysaccharide treated cells, $A_{\text{contr.}}$ is the absorbance of control.

Data were expressed as means $\pm$ standard deviations (SD) of three replicates. Statistical calculations by SPSS version 18.0 software were carried out. One way ANOVA was employed to analyze the statistical differences between the treated groups and control group. P values < 0.05 were considered to be statistically significant.

3. Results and discussion

3.1 Solution conformation of SeASPs

In spite of many merits as biological response modifiers, polysaccharides of nature source suffer from several drawbacks including low solubility and poor bio-activities. Thus, most applications rely on chemical modification of polysaccharides via functionalization (Liu, Willför & Xu, 2015). These chemical reactions have been used to alter the physicochemical properties, solubility and solution conformation. More importantly, the evolution of solution conformation during chemical modification will affect the biological activities of functionalized polysaccharide (Chen et al., 2014a). The conformation of soluble polysaccharides can

be calculated using multi-angle light scattering technique that outline the solution behavior of polymers as a function of solution concentration and scattering angle. For polysaccharide in dilute solution, the conformation information of chains results in the fractal dimension (d_f), which can be determined by the power law describing the M_w dependence of $\langle S^2 \rangle_z^{1/2}$. It has been reported that the d_f value of 3 for three-dimensional globular shape conformation, 2-3 for branching polymers with compact chain conformation, 1.5-2 for a flexible polymer in a good solvent and 1-1.5 for a rigid rod chain (Peng & Zhang, 2002; Tao, Zhang & Peter, 2006).

Table 1 Se content, weight average molecular weight and d_f values of selenized ASP

Samples	Se content ($\mu\text{g/g}$) ^a	$M_w \times 10^4$ (g/mol) ^b	Relation equation	d_f	Solution Conformation
ASP	-	7.348	$\langle S^2 \rangle_z^{1/2} = 0.203M_w^{0.35 \pm 0.027}$	2.86	between hard sphere and random coil (fully swollen)
SeASP ₁	168	4.363	$\langle S^2 \rangle_z^{1/2} = 0.131M_w^{0.15 \pm 0.03}$	6.67	hard sphere
SeASP ₂	1703	1.141	$\langle S^2 \rangle_z^{1/2} = -1.724M_w^{0.77 \pm 0.02}$	1.29	between rigid rod and random coil
SeASP ₃	623	1.026	$\langle S^2 \rangle_z^{1/2} = -1.22M_w^{0.66 \pm 0.10}$	1.52	random coil
SeASP ₄	969	2.268	$\langle S^2 \rangle_z^{1/2} = 0.46M_w^{0.31 \pm 0.13}$	3.22	hard sphere
SeASP ₅	1210	1.426	$\langle S^2 \rangle_z^{1/2} = -1.19M_w^{0.67 \pm 0.30}$	1.49	between rigid rod and random coil
SeASP ₆	264	5.407	$\langle S^2 \rangle_z^{1/2} = 1.79M_w^{0.07 \pm 0.001}$	- ^c	-

^{a & b} Data from our previous report (Wang et al., 2012)

^c Not detected

As already shown in our previous study, the Se contents and M_w of SeASPs were strongly dependent on reaction conditions (i.e. reaction temperature, HNO_3 concentrations and catalyst amount). The Se contents of SeASPs varied from 168 to 1703 $\mu\text{g/g}$ (Wang et al., 2012). In the present study, it was important to study how M_w or Se content affects its conformation in aqueous solution. Table 1 summarized the data of Se content, M_w , straight line fitting and d_f values of selenized ASP (SeASP₁₋₆). The plot of M_w versus $\langle S^2 \rangle_z^{1/2}$ was illustrated in double logarithmic coordinates (Fig. 1A). As shown in our published article, the d_f value of ASP was calculated to be 2.86, indicating a conformation between hard sphere and fully swollen branched polymer in aqueous solution (Wang et al., 2009). After selenylation, all SeASPs showed decreased M_w ($1.026\text{--}5.407 \times 10^4$ g/mol) due to the degradation of polysaccharide chains in HNO_3 . The d_f values of SeASP₂ and SeASP₅ were 1.29 and 1.49, which lie in the range between rigid rod and random coil. SeASP₃ with a low M_w of 1.026×10^4 g/mol exhibited a solution conformation of random coil (d_f value of 1.52). Whereas, SeASP₁ and SeASP₄ having high M_w possessed higher d_f values of 3.22 and 6.67, respectively. As can be seen from Table 1, a decrease in d_f values revealed that SeASPs with different conformational features that were highly dependent on M_w . These results indicated that SeASPs exhibited a more rigid conformation in low molecular weight range and compact spherical conformation in high molecular weight

range, which was likely as a result of some aggregations presented in higher molecular weight samples (Fig. 1B). Similar result was reported that selenized pectic polysaccharide (isolated from *Codonopsis pilosula*) exhibited a linear random coil with the Se content of 478.17 $\mu\text{g/g}$ (Chen et al., 2014b). Mao et al reported a globular conformation with random coil structure of Se-GP11 which was extracted and purified from Se-enriched *Grifola frondosa* (Mao et al., 2016).

Fig. 1 Solution conformation of SeASPs. (A) Log-log plot of M_w versus $\langle S^2 \rangle_z^{1/2}$ of ASP and SeASPs. (B) Schematic representation of solution conformation transition after selenylation.

It was in accordance with our previous reports that modified polysaccharides with lower M_w could achieve a more rigid conformation than high M_w polysaccharides (Wang et al., 2014). One of the possible reasons was that the anionic groups such as $-\text{SeO}_2\text{H}$, $-\text{SO}_3\text{H}$ and $-\text{PO}_3\text{H}_2$ enhanced the steric hindrance of the modified polysaccharide chains due to the elastic contributions. The relatively low d_f values of sulfated and phosphorylated derivatives from *Rhizoma Panacis Japonici* also supported this (Chen et al., 2014a). However, Se content had very little influence on d_f values in the current study (Table 1). This could be due to the fact that Se content ($\mu\text{g/g}$) was much lower than sulfur and phosphorus content (mg/g) in modified polysaccharides. More specifically, the pK_a value of $-\text{SeO}_2\text{H}$ is relatively higher than $-\text{SO}_3\text{H}$. Such a feature could be assigned to the fact that, in selenized polysaccharides with low Se content ($\sim 5000 \mu\text{g/g}$), intra-molecular repulsive interaction caused by $-\text{SeO}_2\text{H}$ was negligible. Zhang and colleagues provided that only the rod-like chains of lentinan with low molecular weight (ultrasonic method) could form ordered aggregates as a result of the strongest interaction and the smallest steric hindrance between each chain (Zhang, Li & Zhang, 2010). It could be concluded that the solution conformation of SeASP was then mainly governed by chain constitutes and molecular weight.

3.2 Analysis of monosaccharide composition

It is well accepted that monosaccharide composition is one of the most important factors to determine the physicochemical characteristics and biological activities of heteropolysaccharides. Fig. 2 showed the monosaccharide composition analysis of ASP and SeASP₂. The presence of the monosaccharides in all samples were confirmed by comparison with the result of the acetyl derivatives of standard monosaccharides (Fig. 2A). As can be seen from Fig. 2B, ASP was composed of five monosaccharides: arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc) and galactose (Gal) with the molar ratio of 1:4.2:45.9:9.7:11.4. ASP had much higher mannose content, making it the predominant monosaccharide component. The molar ratio of Man/Glc and Man/Gal was 4.7 (45.9/9.7) and 4 (45.9/11.4), respectively. After modification, five peaks were identified as Ara, Xyl, Man, Glc and Gal. However, an obvious difference in molar ratio of SeASP₂ (1:18.2:34.8:39.2:13.2) was found. As shown in Fig. 2C, the dominant components of SeASP₂ were determined to be Glc and Man. The molar ratio of Man/Glc and Man/Gal was calculated as 0.88 (34.8/39.2) and 2.6 (34.8/13.2), respectively. A dramatically decrease in Man content was likely due to the acid hydrolysis of backbone during selenylation (*Section 3.1*). It had been shown by our earlier reports that the acidic hydrolysis with esterification reagent such as chlorosulfuric acid could be observed with the detection by means of SEC analysis (Wang et al., 2010; Wang et al., 2015).

Fig. 2 Chromatograms of polysaccharides degraded by TFA (A) standard monosaccharides, (B) ASP and (C) SeASP₂.

3.3 Polysaccharide-congo red complexes formation

It is well known that the triple-helix conformation of polysaccharide (such as glucan and curdlan) can be identified by a bathochromic shift in the maximum absorption values ($\lambda_{\max}$) of the polysaccharide-CR complexes in visible spectrum

(Guo et al., 2016; Liu et al., 2016). The evolution of $\lambda_{\max}$ of CR solution and SeASP₂-CR complexes at various concentrations of NaOH solution were shown in Fig. 3. CR solution showed the $\lambda_{\max}$ at 494 nm. The effect of SeASP₂ concentrations on the $\lambda_{\max}$ of SeASP₂-CR complexes was shown in Fig. 3A. The $\lambda_{\max}$ of SeASP₂-CR complexes increased with the increasing of SeASP₂ concentration from 0.2 to 2 mg/mL. It indicated that SeASP₂ and CR formed complex with a bathochromic shift of 18 nm. An equilibrium state between SeASP₂ and CR was shown when polysaccharide concentration increased up to 1-2 mg/mL. It could be also assumed that all the positively charged CR molecules were saturated by $-\text{SeO}_2^-$ groups (Fig. 3B). The result was similar to that for the report of Grifolan (extracted from the fungus *Grifola frondosa*), indicating the existence of rigid conformation of SeASP₂ (Mao, Hsu & Hwang, 2007).

To investigate the conformation transition of SeASP₂-CR complex, the $\lambda_{\max}$ in the presence of various concentrations of NaOH was recorded at the polysaccharide concentration of 2 mg/mL (Fig. 3C). The $\lambda_{\max}$ of SeASP₂-CR complex decreased gradually and close to 494 nm when the concentration of NaOH higher than 1 M. It was reported that linear or branched glucans and curdlan had a triple-helical conformation in aqueous or weakly alkaline solution (Liu et al., 2016; Mao, Hsu & Hwang, 2007). The descending behavior of the $\lambda_{\max}$ for SeASP₂-CR complex was shown in low NaOH concentration. It was indicated that SeASP₂ showed a rigid conformation as evidenced in SEC-MALLS analysis. However, no triple-helical conformation was observed in polysaccharide-CR binding assay. Our earlier research

and Yi et al reported similar results that sulfated ASP with C-2 and C-3 substitution and longan pulp polysaccharide exhibited a transition from rigid to flexible conformation in polysaccharide-CR interactions (Wang et al., 2016; Yi et al., 2012). The proposed mechanism of conformation transition in presence the of NaOH solution might be due to the breakage of inter/intra-molecular hydrogen bonds of SeASP₂. Moreover, the electrostatic interaction between SeASP₂ and CR might be disrupted by NaOH and caused the de-aggregation of SeASP₂-CR complex (Fig. 3D).

Fig. 3 Complex formation with congo red (A) Plot of the maximum absorption values of SeASP₂-CR at different concentrations of polysaccharide at 20°C. (B) Electrostatic interaction between SeASP₂ and CR. (C) Plot of the maximum absorption values of SeASP₂-CR at various concentrations of NaOH at 20°C. (D) The effect of NaOH on the conformational transition of SeASP₂-CR complex.

3.4 AFM observations

AFM is a useful tool for observing the surface and topography of soft biological surface such as proteins, polysaccharides, DNA and the interactions between biopolymers and particles (Marshall et al., 2009). The surface morphology of SeASP₂ at different concentrations was shown in Fig. 4. In this study, molecular height in section analysis was employed to measure the size of polysaccharide molecular due to the tip broadening effects (Marshall et al., 2009). The obtained image showed that SeASP₂ appeared spherical morphologies linked with worm-like chains under a relatively higher concentration of 20 µg/mL (Fig. 4A). The height of the spherical lumps with an average value of 8.2 nm was obtained by particle analysis (the minimum value of 4.7 nm and the maximum value of 13.1 nm). The worm-like chains had a height distribution about 1 nm by using section analysis (Fig. 4B). The height of SeASP₂ suggested that molecular aggregation was involved, which was ascribed to the -OH providing inter/intra-molecular interactions (Kong et al., 2015). Chen et al also reported the aggregates of β -(1→3)-D-glucan in AFM analysis (Chen et al., 2016).

Fig. 4 The topographical AFM image of SeASP₂ at different concentrations (A) 2D image of 20 µg/mL. (B) Section analysis and 3D image of 20 µg/mL. (C) 2D image of 10 µg/mL. (D) Section analysis and 3D image of 10 µg/mL. (E) 2D image of 1 µg/mL. (F) Section analysis and 3D image of 1 µg/mL.

Fig. 4C showed the topographical AFM image of SeASP₂ at the concentration of 10 µg/mL. Compared to Fig. 4A, a clear difference in the morphology of single rod-

shaped chains with different lengths was observed. Section analysis result showed that the height ranged from 0.64 to 1.11 nm (Fig. 4D). It was deduced that the molecular aggregation of SeASP₂ was weak compared with that of higher concentration. After dilution to a lower concentration of 1 µg/mL, the extended short chains without branches of SeASP₂ were observed in Fig. 4E, indicating the morphology of the worm-like chains. The average height of all observed chains was around 0.6 nm in section analysis (Fig. 4F) and no molecular aggregation was observed. Thus, single molecular morphology of SeASP₂ was obtained in a concentration dependent manner. The molecular morphology transition from linear and tangled network structures (Zhang et al., 2007) to an extended stiff chain conformation was found after selenylation. It was in good agreement with the results obtained by SEC-MALLS (*Section 3.1*), CR (*Section 3.3*) assay and our earlier report that lower *M_w* might lead to a rigid conformation in acid environment (Wang et al., 2016). Average molecular weight is a key factor to influence the molecular conformation of modified polysaccharides.

3.5 Anti-tumor activity in vitro

Anti-tumor activities of polysaccharides differ greatly in their physical properties, chemical structure and advanced structure (Meng, Liang & Luo, 2016), such as solubility, primary structure (average molecular weight, monosaccharide residues, position of glycosidic linkages and the sequence of monosaccharide residues) and solution conformation (triple helix, single helix and random coils). The anti-tumor and

immunomodulation activity could be enhanced by chemical modification (i.e. sulfation, phosphorylation and selenylation) of polysaccharides (Huang & Zhang, 2011; Gao et al., 2016; Chen et al., 2014b; Jiang et al., 2015). However, the correlation between structure aspects and anti-tumor activity was unclear. Some low M_w polysaccharides (such as schizophyllan) exhibited similar anti-tumor activities compared to polysaccharides with higher M_w (Meng, Liang & Luo, 2016). Moreover, phosphated glucan from *Poria cocos* mycelia presented stronger anti-tumor activities due to its expanded chain conformation after modification (Huang & Zhang, 2011). In order to study the effect of selenylation on the bioactivity of modified polysaccharides, ASP and SeASP₂ were tested in the CCK-8 assay to assess its anti-tumor potential in human hepatocellular carcinoma HepG-2 cells, lung adenocarcinoma A549 cells and cervical squamous carcinoma Hela cells in vitro.

As we know, tumor inhibition rate is usually used as an index for screening the anti-tumor drug. The antiproliferative activity of ASP and SeASP₂ on three cell lines was shown in Fig. 5. ASP exhibited a dose-dependent activity against HepG-2 cells at the concentration range of 25-200 $\mu\text{g/mL}$ (Fig. 5A). However, the highest inhibition rate of ASP was only 12.9%. No obvious anti-tumor activities and dose-dependent features against A549 cells and Hela cells were observed with ASP at the tested concentration ranges (Fig. 5B and 5C). It was reported that if the inhibition rate higher than 10%, the corresponding concentration was considered to be cytotoxic (Wu et al., 2016). This result appeared that in this concentration range, no significant antiproliferative activity was observed in ASP. After selenylation, SeASP₂ markedly

promoted anti-tumor activity against HepG-2 cells, Hela cells and A549 cells with a dose-dependent manner. At the concentration of 200 $\mu\text{g/mL}$, the inhibition rates on HepG-2 cells and Hela cells were 35.1% and 47.4%, respectively (Fig. 5A and 5C). Compared with those of HepG-2 cells and Hela cells, there was a significant increase of inhibition rate for A549 cells. The tumor inhibition rate was 28.5, 46.7 and 62.2% at the concentrations of 100, 150 and 200 $\mu\text{g/mL}$, respectively (Fig. 5B). The IC_{50} value was calculated to be 161.6 $\mu\text{g/mL}$ according to the relation between sample concentrations and inhibition rates. These results demonstrated that the selenized derivatives showed much higher inhibitory activity against all three cells compared to the native ASP, indicating that the anti-tumor activity could be enhanced by selenylation.

Many reports had indicated that the substitution groups of modified polysaccharides usually had a close relationship with their biological activities (Meng, Liang & Luo, 2016; Abu et al., 2015). It had also been documented that the anti-tumor activities of selenized polysaccharides depended on its Se content. Ji et al reported that seleno-*Capparis spionosa* polysaccharide (Se-CSPs, Se content of 5518 $\mu\text{g/g}$, molecular mass data was not given) obtained from selenylation could significantly increased cytotoxicity to human gastric cancer SGC-7901 cells with the IC_{50} value of 116 $\mu\text{g/mL}$ (Ji et al., 2012). A selenized pectic polysaccharide from *Codonopsis pilosula* (sCPP1b, molecular mass data was not given) with low selenium content of 478.17 $\mu\text{g/g}$ was obtained in $\text{HNO}_3\text{-Na}_2\text{SeO}_3$ method. After selenylation, sCPP1b exhibited stronger cancer growth inhibition rate in vitro. At the concentration of 200

$\mu\text{g/g}$, the inhibition rates were about 43% on Hela cells, 42% on BGC-823 cells and 60% on A549 cells, respectively (Chen et al., 2014b). It suggested that in the same Se content ranges, SeASP₂ showed similar inhibition rate compared to Se-CSPS and sCPP1b. However, the effect of molecular weight and solution conformation on the anti-tumor activity should not be neglected. As talked above, SeASP₂ with low M_w showed a more expanded conformation between rigid rod and random coil, which was attributed to the hydrolysis of polysaccharide chains in acid environment. Wei and colleagues proved that selenized *Radix hedysari* polysaccharides (Se-RHP) showed a significant decrease in M_w from 27.7 kDa to 62.7 kDa in selenylation. Se-RHP showed greater antioxidant activities and neuroprotective effects against A β_{25-35} -induced oxidative stress and apoptosis in SH-SY5Y human neuroblastoma cell (Wei et al., 2015). It was possible that the bioactivity of Se-containing polysaccharides was not a function of a one factor but a multiple action of many factors, such as Se content, M_w and solution conformation.

Moreover, some natural occurred Se-containing polysaccharide also exhibited improved anti-tumor activities in vitro. Shang et al reported a novel Se-containing polysaccharide (SeGLP-2B-1) from the Se-enriched mycelia of *Ganoderma lucidum*. The Se content and average molecular mass was 186.7 $\mu\text{g/g}$ and 1.06×10^6 Da, respectively. Compared to the native GLP-2B-1 (with a similar molecular mass of 0.93×10^6 Da), SeGLP-2B-1 exhibited an approximately 10-fold stronger antiproliferative activity against six human cancer cell line (Shang et al., 2009). A Se-polysaccharide (Se-ZYTP) was isolated from Se-enriched Ziyang green with the

Se content of 2.14 $\mu\text{g/g}$ (molecular mass data was not given). After 48 h cultivation with Se-ZYTP, the cell viability in human osteosarcoma U-2 OS cells was 31.4% and 21.2% for 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$, respectively (Wang et al., 2013). Contrary to chemically selenized polysaccharides, the molecular weight profile of both Se-containing polysaccharide and the native polysaccharide (without submerged in Se-enriched medium) was similar. Therefore, the introduction of Se into polysaccharide could significantly enhance its anti-tumor activity, further suggesting that Se content played an important role in the bioactivity of selenized polysaccharides. Thus, further investigation is necessary to clarify the relations between structure features and bioactivity in selenized polysaccharides.

Fig. 5 Antiproliferative activity of ASP and SeASP₂ (48 h) against HepG-2 cells (A) A549 cells (B) and Hela cells (C). The inhibition rates were presented as mean values (n=3). *P<0.05 and **P<0.01 compared with the control. #*P*<0.05, ##*P*<0.01 and ###*P*<0.001 compared with ASP.

4. Conclusion

In summary, selenized ASP was synthesized by $\text{Na}_2\text{SeO}_3/\text{HNO}_3/\text{BaCl}_2$ with the Se contents varied from 168-1703 $\mu\text{g/g}$. After selenylation, SeASPs showed decreased M_w ($1.026\text{-}5.407\times 10^4$ g/mol) due to the degradation of polysaccharide chains in HNO_3 , which was supported in monosaccharide composition analysis. Meanwhile, a decrease in d_f values revealed that SeASPs with different conformational features that were highly dependent on M_w . SeASPs exhibited a more rigid conformation in low molecular weight range and compact spherical conformation in high molecular weight range. The solution conformation of SeASP was mainly governed by molecular weight. CR binding experiment and AFM results also supported the idea of the rigid conformation in aqueous solutions.

Moreover, SeASP₂ exhibited greater antiproliferative activity against HepG-2 cells, A549 cells and Hela cells in concentration-dependent fashions as determined by CCK-8 assay in vitro. The IC_{50} value was calculated to be 161.6 $\mu\text{g/mL}$ on A549 cells. This result indicated that the antiproliferative activity of ASP against tumor cells could be enhanced by selenylation. Further research on the synthesis of SeASP with higher Se content is needed in order to expose more details on solution conformation and anti-tumor activities.

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