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Novel vanadyl complexes of alginate saccharides: Synthesis, characterization, and biological activities

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DPPH (PubChem CID: 2735032)
Sulforhodamine B (PubChem CID: 9916275)
p-Nitrophenol phosphate (PubChem CID: 255)

ABSTRACT

Vanadium compounds present many physiological functions. However, vanadium(IV) and (V) salts are difficult for gastrointestinal absorption and have strong side effects. Therefore organic oxovanadium compounds gain more attention. Vanadyl alginate polysaccharides (VAPS) and vanadyl alginate oligosaccharides (VAOS) were obtained from aqueous solutions of $VOSO_4$ at pH 12. They were characterized by infrared spectroscopy, UV–vis spectroscopy and inductively coupled plasma-mass spectrometry (ICP–MS). The antioxidant activity of oxovanadium(IV) complexes was investigated in hydroxyl and DPPH radical scavenging systems *in vitro*. The results reveal that activities of VAPS and VAOS in the two systems were stronger than those of alginate polysaccharides (APS) and alginate oligosaccharides (AOS), respectively. In addition, VAPS and VAOS promoted significantly the antiproliferation of ligands of human hepatoma cell line BEL-7402. Oxovanadium(IV) complexes were potent inhibitors of protein tyrosine phosphatase 1B (PTP1B) with IC_{50} values in the range of 6.4–18.7 μg/mL, indicated in biochemical assays. In addition, Vanadyl-alginate had no significant side effects on proliferation and viability of HL-7702 hepatic cells. In the future, they can be added to medicines and ease the growing threat that cancer and diabetes mellitus cause to human health.

1. Introduction

Vanadium(V), a transition metal element (Etcheverry, Williams, & Baran, 1997) is essential for physiological functions of human beings and animals. Vanadium ions and complexes are found with potent insulin-like effects (Evangelou, 2002), anti-diabetic activities (Baran, 2004), and anti-cancer activities (Ashiq et al., 2008). Moreover, several recent studies show that many vanadium compounds present significant antioxidant activities (Ashiq et al., 2008; Etcheverry et al., 2008; Mohammadi & Yazdanparast, 2010; Naso et al., 2011) *in vitro* and *in vivo*. However, vanadium(IV) and (V) salts are difficult for gastrointestinal absorption and have strong side effects (Li, Ding, Baruah, Crans, & Wang, 2008) indicated in biochemical assays. Therefore, synthesis of novel vanadium complexes with organic chelating ligands has become a scientific task and their biological and pharmacological properties have been evaluated and reported in animal and cell models (Thompson et al., 2004; Thompson & Orvig, 2006).

Alginate, a natural acidic linear polysaccharide (Gacesa, 1988), consists of α-L-guluronate and β-D-mannuronate with 1 → 4 glycosidic linkages. It occurs in the cell walls of seaweed and can be synthesized with some bacteria (Costerton, 1999). Due to their variable properties, alginate and its derivatives have been widely used in food, biotechnology, pharmaceutical and cosmetic industry (Iwamoto et al., 2005; Yamamoto, Kurachi, Yamaguchi, & Oda, 2007). However, its high molecular weight limits the application in many fields. Recent reports show that, low molecular weight polysaccharide and oligosaccharide prepared from alginate feature many biochemical activities, including antioxidant activity (Tusi, Khalaj, Ashabi, Kiaei, & Khodagholi, 2011; Wang

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et al., 2007), anti-hypersensitive activity (Burana-osot et al., 2009), anti-allergy property (Uno, Hattori, & Yoshida, 2006), enhancing protection against infection with some pathogens (Tusi et al., 2011) and advanced glycation end-products (AGEs) inhibitory effect (Sattarahmady, Khodagholi, Moosavi-Movahedi, Heli, & Hakimelahi, 2007).

In this work, VAPS and VOAS were two new coordination compounds, therefore it was necessary to confirm their surface chemical structure. According to biological-pharmacological effects that vanadium compounds may have, the anti-proliferative activities of VAPS and VAOS onto human hepatoma cell line BEL-7402 and the ability against PTP1B were tested. Then they can contribute to ease the growing threat that cancer and diabetes mellitus cause to human health. Their antioxidant effects were tested using hydroxyl radical and DPPH radical models. To study the anti-proliferative activities of VAPS and VAOS onto human hepatoma cell line BEL-7402, tests were carried out by the sulforhodamine B (SRB) method. To understand their ability against PTP1B, the main negative regulatory factor in insulin signaling pathways, tests were carried out as per Ryszard et al. (Gryboś, Paciorek, Szklarzewicz, Matoga, Zabierowski, & Kazek, 2013). Besides, MTT assay was conducted to test their cytocompatibility.

2. Experimental sections

2.1. Materials

Alginate was purchased from Sinopharm Chemical Reagent Co Ltd (China). All other reagents were in analytical grade and used without further purification. IR spectra were measured on a Jasco-4100 FT-IR spectrometer with KBr disks. The content of vanadium was determined by ELAN DRC II ICP–MS. The average molecular weight of the alginate saccharides were determined by DNS methods (Costantino et al., 1999).

2.2. Preparation of APS and AOS

Alginate polysaccharides/alginate oligosaccharides were prepared by acid hydrolysis (Zhang et al., 2006)/oxidative degradation (Tian, Liu, Hu, & Zhao, 2004; Yang, Li, & Guan, 2004). Sodium alginate (10 g) was dissolved in 500 mL water, using sulfuric acid adjusting the concentration of hydrion to 0.22 mol/L under reflux at 90 °C for 24 h. After regulated reaction was completed, the resulting solution was adjusted to pH 7 with NaOH and filtered using a Buchner funnel under vacuum condition. Hydrolysis alginate solution was purified by hydrogen-SAC (strong acidic cation) exchange resins and anion exchange resins in hydroxylic form. The eluent was concentrated and then precipitated four times with absolute ethanol. APS were dried in a vacuum oven at 40 °C for 24 h.

Sodium alginate (10 g) was dissolved in 200 mL water, adding 20 mL 30% H_2O_2 under reflux at 100 °C for 50 min. After regulated reaction was completed, the resulting solution was adjusted to pH 7 with NaOH and filtered using a Buchner funnel under vacuum condition. The following steps were the same as what had been done for APS. Then AOS was gained.

2.3. Preparation of VAPS and VAOS

Aqueous solution (10 mL) of alginate saccharide (1 g) was prepared by adjusting the pH to 12 using 1 mol/L NaOH and stirring for 2 h at room temperature. Then, 0.25 g $VOSO_4{\cdot}5H_2O$ was dropped slowly into the saccharide solution with constant stirring, and pH of the solution was maintained at 12 during whole process. After incubatiom for 4 h, the solution was concentrated and precipitated four times with absolute ethanol. The obtained oxovanadium alginate saccharides were dried in a vacuum oven at 40 °C.

2.4. Hydroxyl radical scavenging activity

The hydroxyl-radical scavenging activities of alginate saccharides and oxovanadium alginate saccharides were measured according to the method described by Guo et al. (2005), Guo et al. (2007) with slight modification. The reaction solution (total volume 4.5 mL) containing 0.5 mL saccharide (2, 4, 6, 8, 10 mg/mL) was incubated with 1 mL EDTA–Fe^{2+} (0.945 mM), 1 mL safranine O (80 mg/L), 1 mL·H_2O_2 (0.03%) and 1 mL potassium phosphate buffer (0.15 mM, pH 7.4) for 30 min at 37 °C. Vitamin C was used as the positive control in the experiment. Finally, the absorbance of the reaction solution was measured at 520 nm. The effect of scavenging hydroxyl radicals was calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left[\frac{\left(A_{\text{sample 520 nm}} - A_{\text{blank 520 nm}}\right)}{\left(A_{\text{control 520 nm}} - A_{\text{blank 520 nm}}\right)}\right] \times 100$$

Where $A_{\text{blank 520 nm}}$ was the absorbance of the blank (distilled water instead of the samples), $A_{\text{control 520 nm}}$ was the absorbance of the control (distilled water instead of the sample and EDTA–Fe^{2+}).

2.5. DPPH radical scavenging activity

The DPPH radical scavenging activities of alginate saccharides and oxovanadium alginate saccharides were estimated in triplicate using a modified Yamaguchi Method (Yamaguchi, Takamura, Matoba, & Terao, 1998). An ethanolic solution of DPPH (2 mL, 0.2 mM) was added to 1 mL of the saccharide solutions (2, 4, 6, 8, 10 mg/mL) in 1 mL Tris–HCl buffer (0.1 M pH 7.4). The mixture was left in the darkness at room temperature for 30 min, and then the absorbance of the mixture was measured at 517 nm. Vitamin C was used as the positive control in the experiment. The effect of scavenging DPPH radicals was calculated using the following equation:

$$\text{Scavenging effect}(\%) = \left[1 - \left(\frac{A_{\text{sample}}}{A_{\text{blank}}}\right)\right] \times 100$$

where A_{blank} was the absorbance of the blank (0.1 M Tris–HCl buffer instead of the samples).

2.6. Cell proliferation assay

Anti-proliferative activity was evaluated with human hepatoma cell line BEL-7402 by the SRB method (Iwasa, Moriyasu, Yamori, Turuo, Lee, & Wiegrebe, 2001). RPMI–1640 was used as the culture medium. An amount of 200 μL cell suspension was cultured in 96-cell plates in density of 2×10^5 cells/mL. Different doses of samples were added into each well for incubation 72 h and the final concentration of samples were 16, 63, 250, 1000, and 4000 μg/mL. Then, cells were fixed with trichloroacetic acid (12%) and the cell layer stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm.

$$\text{Inhibition ratio} = \left(1 - \left(\frac{A_{\text{sample}}}{A_{\text{blank}}}\right)\right) \times 100\%$$

A_{sample} is the absorbance of the sample at 515 nm; A_{blank} is the absorbance of the blank at 515 nm.

2.7. PTP1B inhibition assay

PTP1B is a negative regulatory factor in insulin signal transduction. The subdued insulin signal can disorder the glycometabolism in the body. So the inhibition against PTP1B is in favor of insulin signal transduction and it contributes to maintain glycometabolism. Inhibition against PTP1B by VAPS and VAOS was tested, using *p*-nitrophenol phosphate (pNPP) as the substrate, as per Ryszard et al. (Gryboś et al., 2013). The reaction was carried out at 37 °C in black, half-area 96-well plates with human, recombinant PTP1B. MoPs was used as the reaction buffer and the pH was 7.4. VAPS and VAOS were dissolved in DMSO at a concentration of 80 μg/mL. 0.02 Mm PTP1B and 0.01 M *p*-NPP were also added to the assay buffer. The reaction system was incubated for 30 min at 37 °C in water bath. 1 M NaOH was added to terminate the reaction. The fluorescence was measured at 405 nm.

$$\%\text{Inhibition} = \frac{\left(V_{\text{DMSO}} - V_{\text{Sample}}\right)}{V_{\text{DMSO}}}$$

$$\%\text{Inhibition} = \text{Bottom} + \left(\frac{(\text{Top} - \text{Bottom})}{\left(1+10^{(\text{Log IC}_{50}-X)\times h}\right)}\right)$$

where V_{DMSO} was the initial enzyme velocity without samples, V_{Sample} was the initial enzyme velocity with samples. IC_{50} was the half maximal (50%) inhibitory concentration (IC) of a substance, h was the hill index.

2.8. Cell proliferation and viability

HL-7702 hepatic cells were cultured in DMEM supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL) and 0.5% L-glutamine. They were incubated at 37 °C in an atmosphere containing 5% CO_2 and the culture medium was changed every 2 days. HL-7702 cells were isolated into single cells using a suspension of 0.25% trypsin solution. Cell suspension was diluted with fresh medium and plated in 96-well plates at a density of 1×10^4 cells/well. In the experimental groups, compound solutions were added to each well to the concentration of 250 μg/mL and in the control group equal volumes of PBS was added. After 1, 2, 3, 4, 5 days culture, an MTT test (Boccafoschi, Habermehl, Vesentini, & Mantovani, 2005) was performed to quantify the cell viability. Briefly, solutions were removed carefully and each well was washed taice with 500 μL PBS. 100 μL MTT (5 mg/mL) solution was added to each well and incubated for 4 h at 37 °C. The blue formazan reaction product was dissolved by the addition of 0.5 mL DMSO and the solution was placed on a shaker for 10 min. Then the solution was diluted to 2 mL with PBS. The absorbance was measured at 490 nm using UV spectrophotometer.

3. Results and discussion

3.1. Determination of average molecular weight of APS and AOS

The average molecular weight of APS and AOS is 4.1 kDa and 1.6 kDa, respectively estimated by end group analyses.

3.2. Characterization of VAPS and VAOS

FT-IR spectra of APS, VAPS, AOS and VAOS are shown in Fig. 1. By Comparing IR spectrum of VAPS and APS, we find C—O—C absorption peak is shifted to 3432.24 cm^{-1}. $\nu_{as}(COO^-)$ and $\nu_s(COO^-)$ stretching vibrations are changed to 1619.91 cm^{-1} and 1446.35 cm^{-1}, respectively. According to Etcheverry, Williams, and Baran (1994, 1997), the characteristic absorption of VAPS at

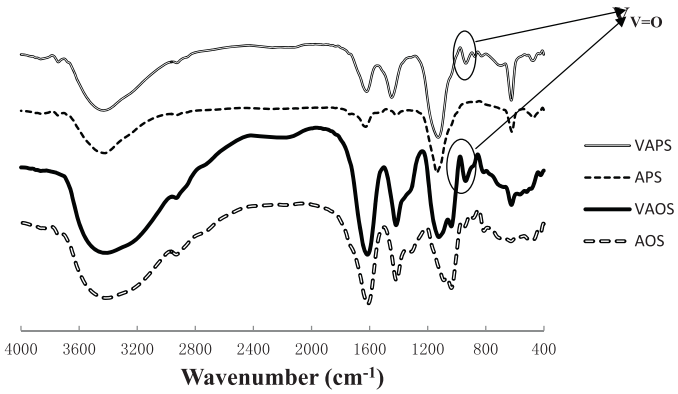

Fig. 1. IR spectra of APS, VAPS, AOS and VAOS.

925.66 cm^{-1} is due to the stretching vibration of V=O. The results suggest the incorporation of alginate polysaccharides with VO^{2+}. The vanadium content of the tested VAPS was 4.3%.

Compared to AOS, the stretching vibration of $\nu_{as}(COO^-)$ and $\nu_s(COO^-)$ in VAOS were shifted to 1616.06 cm^{-1} and 1419.35 cm^{-1}, respectively. C—O—C stretching vibration was changed to 1122.37 cm^{-1} and the band (C—O stretching) at 1037.52 cm^{-1} decreased in intensity. Moreover, the typical V=O stretching vibration was seen at 941.09 cm^{-1}. All these results indicated clearly that the incorporation of alginate oligosaccharides and VO^{2+}. The vanadium content of the VAOS was 3.0%.

Fig. 2 is UV–vis spectra of APS and VAPS and Fig. 3 is UV–vis spectra of AOS and VAOS. They showed two similar bands in pattern characteristic of peroxo complexes (Gabriel et al., 2008; Gabriel et al., 2012). The weak absorption band around 260 nm and the intense absorption band around 220 nm could be due to the electron transition from the π^* orbital of ligands to the vacant d orbitals of vanadium (Gabriel et al., 2008; Sarkar & Mandal, 2000). In addition, the high-intensity band around 220 nm could be associated with π–π^* transition of the ligands and those of *n*–*d* and π^*–*d* from ligands to vanadium ion (Sarkar & Mandal, 2000).

3.3. Scavenging of hydroxyl radical by alginate saccharides

Among reactive oxygen species, hydroxyl radicals are the strongest reactive free radical, which can easily damage amino acids, DNA, membrane components and etc (Liu et al., 2009). Fig. 4 shows positive correlation between concentration and scavenging activity against hydroxyl radical and the samples. APS and AOS exhibited scavenging abilities against hydroxyl radical, with

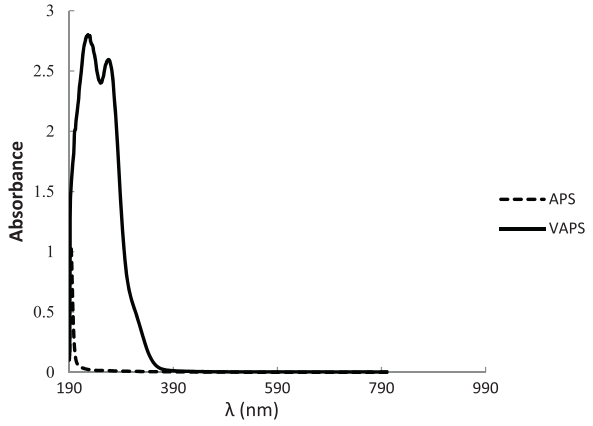

Fig. 2. UV–vis spectra of APS and VAPS.

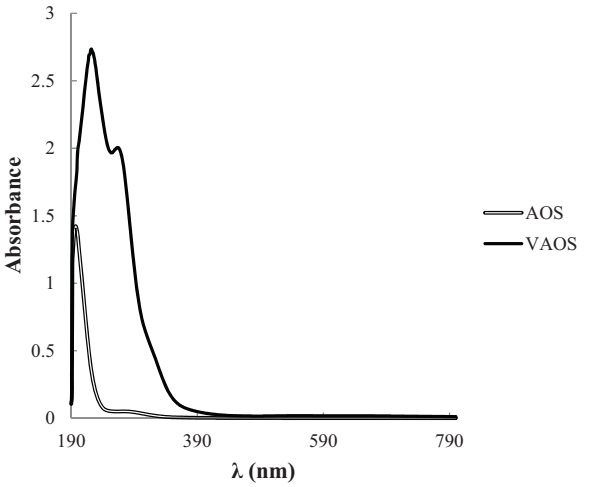

Fig. 3. UV–vis spectra of AOS and VAOS.

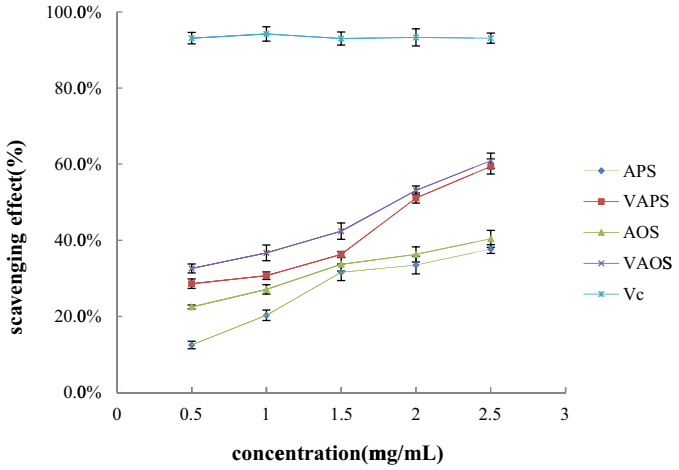

Fig. 5. DPPH radical scavenging effects of alginate saccharides and vanadyl alginate saccharides. Values are means ± SD, $n = 3$.

scavenging effects of 28.5% and 30.6% at 1.11 mg/mL, respectively. Nearly 56.6% inhibition of VAPS was observed at the highest dosage, while it was 58.6% for VAOS. Obviously, the hydroxyl radical-scavenging abilities of VAPS and VAOS were much higher than that of the original alginate saccharides at all the same concentration. According to the previous studies (Shon, 2003; Wang, Zhang, Zhang, & Li, 2008), polysaccharides scavenging hydroxyl radicals are *via* two main mechanisms of (i) chelation with metal ions by alcohol hydroxyl groups against •OH formation, and (ii) cleaning hydroxyl radical by hydrogen-atoms of hydroxyl groups. The scavenging activities of vanadyl alginate saccharides are strongger than those of their ligands, as vanadium is a transition metal element and its d orbital electrons can be easily captured or loss. EPR (electron *para*-magnetic resonance) study revealed that vanadium can maintain balance between V(IV) and V(V) (Willsky, White, & McCabe, 1984). As a result, vanadyl cations can scavenge free radicals by the oxidation reduction in vanadium (Mohammadi & Yazdanparast, 2010). The synergic effects of vanadyl cation and saccharides in the oxovanadium(IV) complexes enhance the hydroxyl radical-scavenging abilities of vanadyl alginate saccharides for much better performance than their ligands do.

3.4. Scavenging of DPPH radical by alginate saccharides

Fig. 5 presents the results of DPPH radical scavenging activities of these saccharides, showing that all of the four samples scavenged DPPH radicals at concentration-dependent manner. In concentration of 0.5–2.5 mg/mL, APS scavenged 12.5–32.7% of DPPH radicals, 28.6–59.4% for VAPS, 22.5–40.4% for AOS, and 32.6–60.9% for VAOS. Therefore, we believe that the vanadyl(IV) cation could improve significantly the antioxidant capacity of alginate polysaccharides and oligosaccharides. It is known that polyphenolic compounds react with DPPH radicals in two ways (Kishk & Al-Sayed, 2007): (i) direct abstraction of phenol hydrogen-atom, (ii) electron transfer from ArOH or ArO^- to DPPH•. Therefore, scavenging of polysaccharides against DPPH radicals could be due to their reduction activities, which may be related to the hydroxyl groups and electron transfer from polysaccharides (ROH or RO^-) to DPPH•. The *d* orbital electrons of vanadium in vanadyl alginate saccharide can be transferred to DPPH•, which improves the scavenging against DPPH radicals of oxovanadium(IV) complexes.

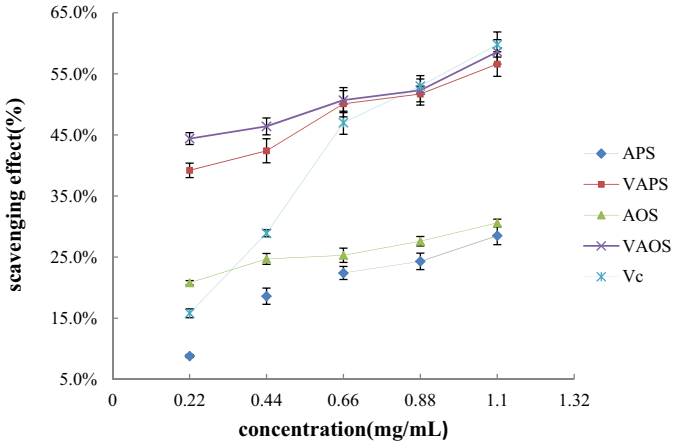

Fig. 4. Hydroxyl radical scavenging effects of alginate saccharides and vanadyl alginate saccharides. Values are means ± SD, $n = 3$.

3.5. Cellular proliferation

The anti-proliferation of the four samples was measured for human hepatoma cell line BEL-7402 in concentration range of 16–4000 μg/mL. Both APS and AOS caused slight inhibitions in cell proliferation in the tested concentration range. However, VAPS and VAOS exhibited strong inhibitory activities against the human cell line. Table 1 shows positive correlation between the inhibitory activity and the two vanadyl complexes in a concentration dependent manner.

3.6. PTP1B inhibition assays by oxovanadium complexes

Results presented that the IC_{50} of VAPS on PTP1B activity was 6.9 ± 0.5 μg/mL, and that of VAOS was 16.5 ± 2.2 μg/mL. Biochemical assays demonstrated that both VAPS and VOAS were potent inhibitors of PTP1B. Vanadium restrained PTP1B *via* two main mechanisms. One was that vanadium acid radical can inhibit the activity of PTP1B by competition, decreasing its dephosphorylation of phosphorylation—tyrosine. The other was that in the cell vanadium could generate reactive oxide species (ROS) by oxidation–reduction. ROS could oxidize cysteine in the domain of PTP1B, forming disulfide bond. The activity of PTP1B was restrained when its active site was changed. These results indicated that both VAPS and VOAS were potent inhibitors of PTP1B.

Table 1
The inhibitory activity in the four samples against hepatoma cell line BEL-7402.

Concentration	Rate of inhibition of APS	Rate of inhibition of VAPS	Rate of inhibition of AOS	Rate of inhibition of VAOS
16 (μg/mL)	1.6 ± 0.20	17.6 ± 0.27	1.5 ± 0.00	0
63 (μg/mL)	0	75.1 ± 0.35	0	79.0 ± 0.31
250 (μg/mL)	3.3 ± 0.16	88.9 ± 1.11	0	85.7 ± 1.37
1000 (μg/mL)	2.6 ± 0.20	90.9 ± 0.58	0	91.2 ± 2.58
4000 (μg/mL)	18.7 ± 0.27	94.3 ± 1.73	0	94.3 ± 1.54

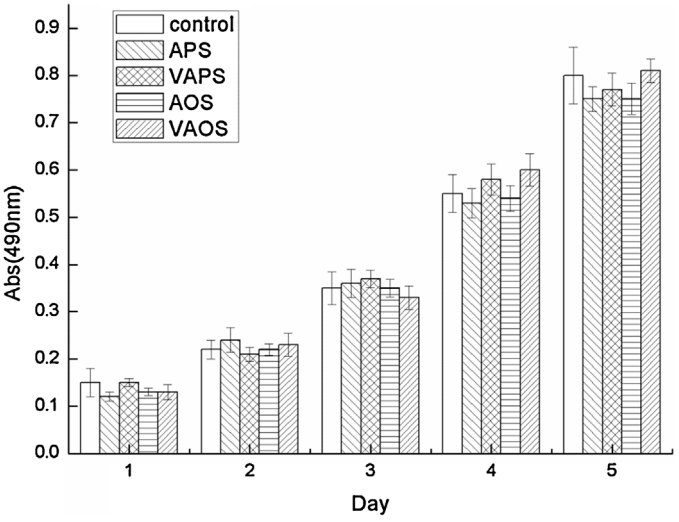

Fig. 6. The viability of HL-7702 hepatic cells measured by MMT assay Values are means ± SD, $n = 6$.

3.7. Cell proliferation and viability

The functional mitochondria can oxidize an MTT solution to give a typical blue-violet formazan which can be solubilized and quantified by spectrophotometer means. This assay is an indirect method of assaying cell growth and proliferation (Yu, Lin, Lin, &, 2006). The influence of VAPS and VAOS on HL-7702 hepatic cells was evaluated by MTT assay. Fig. 6 illustrated the results of the MMT test performed during the 5-day cell proliferation ($n = 6$). Cells are proliferating, as demonstrated by the increased absorbance with time. Compared with the control group, cell proliferations in mediums containing different compounds were no significant difference, which indicated that these four compounds have good cytocompatibility.

4. Conclusion

In this study, we obtained two different oxovanadium(IV) complexes with alginate polysaccharide and oligosaccharide and confirmed that the interaction of oxovanadium(IV) cation with alginate saccharides in infrared spectroscopy, and determined the vanadyl content in ICP–MS. Study on antioxidant activity showed that the hydroxyl radical scavenging activities in oxovanadium complexes were stronger than those of their ligands, and DPPH scavenging activities in oxovanadium complexes could be enhanced by complications. Furthermore, the anti-proliferative activities of VAPS and VAOS toward human hepatoma cell line BEL-7402 and their ability to inhibit PTP1B were studied. Cytocompatibility with HL-7702 hepatic cells were also evaluated by MTT assays. The results suggested that VAPS and VAOS could be explored for pharmaceutical and food industries. More studies are being conducted to test their biosafety using *in vivo* animal models.

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