Chemical Science International Journal

21(2): 1-15, 2017; Article no.CSIJ.39109
ISSN: 2456-706X
(Past name: American Chemical Science Journal, Past ISSN: 2249-0205)

Preparation, Solubilization and *In vitro* Anti-tumour Effect of Water-soluble Betulinic Acid/Oligo(polylvinylamino) Bridged bis(β-cyclodextrin)s Complexes

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YT, XF and SD designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors XF and TW managed the analyses of the study. Authors XF, YH and XY managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/CSJI/2017/39109
Editor(s):
(1) Nagatoshi Nishiwaki, Professor, Kochi University of Technology, Japan.
Reviewers:
(1) Joseph C. Sloop, Georgia Gwinnett College, USA.
(2) Imran Ali, Jamai Millai Islamia Central University, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/23150

Original Research Article

Received 15th October 2017
Accepted 24th January 2018
Published 12th February 2018

ABSTRACT

A series of water-soluble betulinic acid complexes of betulinic acid/oligo(diethylenetriamino) bridged bis(β-cyclodextrin) (BA/DT-β-CD), betulinic acid/oligo(triethylenetetramino) bridged bis(β-cyclodextrin) (BA/TT-β-CD) and betulinic acid/oligo(tetraethylenepentamino) bridged bis(β-cyclodextrin) (BA/TP-β-CD) were prepared and characterized. IR, SEM, MS and ^{1}H NMR were used to characterise these complexes. The results show that the two cyclodextrin cavities and tether groups of the aforementioned bis(β-cyclodextrin)s can form a well-organized pseudo cavity to accommodate the branch group of BA, respectively. By UPLC analysis, the water solubility was

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remarkably increased to approximately 310.97, 144.94 and 75.04 μg mL^{-1} by the solubilising effects of DT-β-CD, TT-β-CD and TP-β-CD, respectively. Furthermore, the anti-tumour effect against HeLa cell line was tested by MTT assay, and the IC_{50} values of BA in BA/DT-β-CD complex water solutions are 103.89 ± 5.12 μmol L^{-1}, which possess an obvious antiproliferative activity against HeLa cell line.

Keywords: Betulinic acid; Bis(β-cyclodextrin); Oligo(polylvinylamino) bridged; water solubility; anti-tumor.

1. INTRODUCTION

Betulinic acid (BA), 3β-hydroxy-lup-20(29)-ene-28-oin acid (Fig. 1), is a triterpenoid present in many plant species, such as white birch bark [1,2], tetracera potatoria (family dilleniaceae) [3] and ziziphus jujubae [4]. The present study shows that the BA is considered as a promising chemotherapeutic agent against HIV infection [5] and has cytotoxicity against melanoma [6], ovarian carcinoma [7] and human breast carcinoma [8] tumor cells. However, it is well-known that a major inconvenience for the future clinical applications of BA is the poor solubility in water (BA, 0.02μg mL^{-1}) [9], which make the bioavailability questionable. Although much effort has been made to improve its water solubility by derivativization [10] or microemulsion technique [11], it is still not possible to sufficiently solve the aforementioned problem.

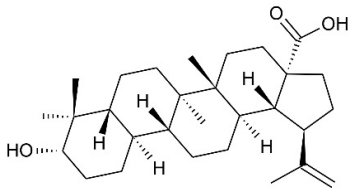

Fig. 1. Betulinic acid

Recently, the technique of molecular encapsulation has been developed to resolve the solubility problem of insoluble drugs [12-22], which has the advantages such as solubility enhancement of highly insoluble drugs [23,24], improvement of bioavailability [25-27], stabilisation of labile drugs [28,29], covering up the taste of drugs [30,31], minimizing harmful side effects [32-35], controlling drug release [36-39] et al.

Cyclodextrins (CDs), a class of hydrophilic natural polymers, have recently attracted attention as the molecular capsule wall materials for insoluble drugs in formulation and delivery systems [40,41]. CDs are torus-shaped cyclic oligosaccharides made up of α-1,4 linked D-glucopyranose with 6(α-),7(β-), and 8(γ-) units, can act as host molecules interacting with appropriately sized guests via their lipophilic center [42,43]. Weng et al. [44] reported that BA molecules can be encapsulated by β-CD, Hydroxypropyl-β-CD, γ-CD and some γ-CD derivatives. The results show that the γ-CD derivatives solubilized BA to much higher extend than β-CD, Hydroxypropyl-β-CD and γ-CD. Besides, Dehelean et al. verifies the antiangiogenic potential [45], anti-skin cancer activity [46] and anti-melanoma activity [47,48] by the inclusion complexes of BA with γ-CD derivatives. Now, however, due to the proper inside cavity and lower production cost, only β-CD and its derivatives have been widely developed in industry [49]. So, the investigation of BA/γ-CD derivative complex for therapeutic application are restricted due to its greater cost than that of β-CD.

In this paper, oligo(polyvinylamino) bridged bis(β-cyclodextrin)s with two hydrophobic cavities and proper nucleophilic tether were synthesized. Upon the complexation of BA molecule, the tether group and the two hydrophobic cavities can form a well-organized pseudo cavity [50] to accommodate the branch group of BA. This pseudo cavity provides a much stronger and more suitable host-guest interaction upon inclusion complexation of BA molecules than β-CD and γ-CD [51]. However, to the best of our knowledge, no scientific study on the preparation, characterization, binding ability, water solubility and anti-tumor effect of BA/oligo(polylvinylamino) bridged bis(β-cyclodextrin)s complexes has hitherto been reported.

2. MATERIALS AND METHODS

2.1 Materials

β-CD was purchased from Aladdin Co., Ltd. (China), recrystallized twice from water and dried for 12 h at 105ºC. BA were obtained from Boylechem Co., Ltd. (China). Betulinic Acid. *p*-Toluenesulfonyl chloride (TsCl, 99%) and

diethylenetriamine (DETA, 99%) were purchased from Aladdin Co., Ltd. (China). The chromatographically pure acetonitrile and methanol were also obtained from Aladdin Co., Ltd. (Shanghai, China). RPMI-1640 medium and fetal bovine serum were supplied from Gibco BRL (USA). l-glutamine, penicillin and streptomycin were purchased from Sigma Chemical Co. Ltd (USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was obtained from Amresco Inc. (USA). The human cancer cell line HeLa was obtained from the Tumor Center (China). Deionized water was prepared by Milli-Q century system (Millipore, American). N,N-Dimethylformamide (DMF) and dimethylsolfoxide (DMSO) was dried over calcium hydride for 2 days and then distilled under a reduced pressure prior to use. HeLa cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and incubated at 37°C in a 5% CO_2 incubator. Every 4–5 days, the cells were subcultured by total replacement using 0.25% (w/v) trypsin in order to maintain activity.

2.2 Instruments

IR samples were recorded by Nicolet IS10 spectrometer (Thermo Fisher Scientific, USA). Thirty-two scans for each sample was operated at room temperature with resolution of 4 cm^{-1}, and the scan scope was in the range of 4,000–400 cm^{-1}.

SEM photographs were determined on JEOL JSM-7500F (Japan). The powders were previously fixed on brass stub using double-sided adhesive tape and then were made electrically conductive by coating in a vacuum with a thin layer of gold. The pictures were taken at an excitation voltage of 5 or 15 kV and a magnification of 2000×.

NMR spectra were performed on API Bruker Avance 400 MHz at 25ºC spectrometer using $SiMe_4$ as the internal standard, using DMSO as the solvent agent.

Elemental analysis was performed on Elementar vario EL cube. The precision of C, H and N analysis were lower than 0.1%.

2.3 Synthesis of oligo(polyvinylamine) Bridged bis(β-cyclodextrin)s

Mono[6-O-(p-tolylsulfonyl)]-β-cyclodextrin (6-OTs-β-CD) [52]. 6-OTs-β-CD was prepared according to previous reports. Synthetic process was listed as follows: 24.0 g (21.2 mmol) of β-CD was dissolved in 200 mL of 6.5 mol L^{-1} NaOH solution, and then 4.0 g (21.2 mmol) of TsCl in 12 mL acetonitrile was added dropwise over 8 min. After 2 h of stirring at 23 ºC, the precipitate formed was collected by suction filtration, and the filtrated was stored overnight at 5 ºC for further precipitation. Finally, 2.9 g of pure white solid was afforded after all the precipitates drying for 12 h in vacuo at 55 ºC. The yield was 11%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.75 (d, 2H), 7.40 (d, 2H), 5.87-5.58 (m, 14H), 4.82 (br s, 4H), 4.78 (br s, 3H), 4.55-4.13 (m, 6H), 3.74-3.43 (m, 28H), 3.42-3.18 (m, overlaps with HOD), 2.38 (s, 3H); ^{13}C NMR (75.47 MHz, 0.1 mol dm^{-3} HCl–D_2O) δ 144.9 (s), 132.8 (s), 129.8 (d), 127.8 (d), 102.1 (m), 81.8 (d), 73.3-71.4 (m), 70.0, 68.7, 59.5 (t), 21.1 (q); elemental analysis calcd (%) for $C_{45}H_{80}O_{34}N_3 \cdot 2H_2O$ (1242), found: C 43.64, H 6.09, S 2.38.

DETA-β-CD. DETA-β-CD was prepared based on the report of May [53]. In brief, 2.0 g (1.59 × 10^{-3} mol) of 6-OTs-β-CD was dissolved in 5 mL of dry NMP and warmed to 70ºC, and then 0.025 g of KI and 0.73 g (5.06 × 10^{-3} mol) of diethylenetriamine were added to the aforementioned mixture. The reaction was stirred for 7 h at 70ºC under the protection of nitrogen. The resultant light yellow solution was cooled to room temperature and diluted with 100 mL of ethanol, the causing precipitate was collected by suction filtration. In washing procedure, the precipitate was firstly dissolved in 10 mL of water, and then filtered over a 0.45 μm cellulose acetate membrane. The filtrate was poured into 50 mL of ethanol and the precipitate was collected. The washing procedure was repeated three times.

After washing, the crude product was purified on a column (4.5 × 7 cm) of 724 resin (H^+ form, 16-50 mesh, Zhengzhou, China) with 400 mL of water and 500 mL of 1 mol L^{-1} aqueous ammonia as eluent, respectively. Fractions containing DETA-β-CD were combined and evaporated to dryness under vacuum. The residue was dissolved in water and the solution evaporated under reduced pressure to remove excess ammonia, this procedure was repeated at least three times. After the residue was dried in vacuo, a pure sample was obtained in 75% yield. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.80-4.52(br, 27H, H_1, 2, 3, 6-OH), 4.05-3.71(br, 28H, $H_{3,5,6}$), 3.56-3.45(br, 14H, $H_{2,4}$), 3.17-2.68(m, 12H, -CH_2, N-H) (Fig. 6); ^{13}C NMR (75.47MHz, 0.1 mol dm^{-3} HCl–

D_2O) δ 101.1 (m), 80.3-80.0 (m), 74.0-73.0 (m), 61.6 (m), 50.5 (s), 48.7 (d), 46.7 (s), 40.2(s), 32.7 (s); elemental analysis calcd (%) for $C_{45}H_{80}O_{34}N_3$. $2H_2O$ (1242): C 43.48, H 6.76, N 3.38, found: C 44.12, H 6.51, N 3.42.

N oligo(polylvinylamino) bis(β-cyclodextrin)s [54]. The mixture of amino β-CD derivatives (1.2 g, 9.8×10^{-4} mol) and 6-OTs-β-CD (1.3 g, 9.9×10^{-4} mol) was allowed to react in 50 mL of DMF and was stirred under nitrogen at 80ºC for 3 d. The resultant solution was poured into 300 mL of acetone, and the formed precipitate was collected by filtration. This procedure was repeated three times. The crude product thus obtained was subsequently purified on a CM Sephadex C-25 ionic column with 1 mol L^{-1} aqueous ammonia as eluent and a Sephadex G-25 column with water as eluent, respectively. After the residue was dried in vacuo, a pure sample was obtained in 15% yield. The synthetic route is shown in the Fig. 2. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.95 (m, 14H, H1), 4.10~3.62(br, 56H, H3, 5, 6), 3.58~3.23(br, 28H, H2,4), 3.0~2.5(m, 11H, -CH_2, N-H); ^{13}C NMR (75,47MHz, 0.1 mol dm^{-3} HCl–D_2O) δ 102.8 (s), δ 80.2 (s), δ 74.2 (s), δ 73.1 (s), δ 60.5 (s), δ 50.1 (s), δ 48.5 (s); elemental analysis calcd (%) for $C_{88}H_{149}O_{68}N_3 \cdot 2H_2O$ (2371): C 44.54, H 6.28, N 1.77, found: C 44.30, H 6.31, N 1.82.

2.4 Preparation of Solid State BA/oligo(polylvinylamino) bis(β-cyclodextrin)s Complex

2.0 mmol of BA and 2.0 mmol of oligo(polylvinylamino) bis(β-cyclodextrin)s were mixed in 15 mL of deionized water and stirred for 3 d at room temperature. The solid residue was then separated by centrifugation at 12,000 rpm for 15 min and the supernatant liquid was filtered over a 0.45 μm cellulose acetate membrane. The solution was then dried by lyophilization and the resulting solid inclusion complex (yield 90%) was collected.

2.5 Solubilization Experiment

The solubility of resulting complexes was performed by saturated solution method. 0.5 g of BA complex (BA/DT-β-CD, BA/TT-β-CD or BA/TP-β-CD) was placed in a centrifuge tube containing 5 mL of water (ca. pH 6.0) for preparing supersaturated solution. Four tubes of same BA complex supersaturated solution were ultrasonic stirring for 0.5 h, 1.0 h, 1.5 h, and 2.0 h at room temperature, respectively. The filtrate was dried by lyophilization and the resulting solid inclusion complex was collected. Then the resulting solid inclusion complexes of BA/DT-β-CD, BA/TT-β-CD and BA/TP-β-CD were performed by the following process. Firstly, a mixture of 80 mL ethanol and resulting solid inclusion complex was treated for 30 min in the ultrasonic oscillator. Secondly, the suspension was separated by centrifugation under 6000 rpm for 10 min, and then diluted to 100 mL by ethanol. Thirdly, the precipitate was collected and the aforementioned extracting procedure was repeated twice. BA extraction solution was obtained by combing all the suspensions, and the suspensions were detected by MS and UPLC, respectively. The solubility of compounds of BA were obtained from the analysis of UPLC quantification.

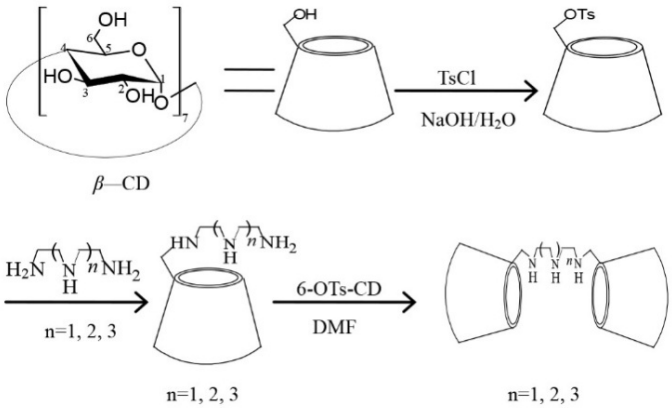

Fig. 2. Syntheses of the oligo(polylvinylamino) bridged bis(β-cyclodextrin)s

2.6 UPLC Quantification

UPLC analysis was performed on Waters Acquityh Class chromatograph. The column used is Acquity Uplc Beh C18 column (2.1 × 50 mm i.d., 1.1 μm, Waters) and the injection volume was 5 μL. The column oven was thermostatted at 25 ºC. The flow rate was 0.25 mL min^{-1} and the detective wavelength was 213 nm. Mobile phase was composed of A (acetonitrile)-B (water): 0.7 min, 60% A, 0.1 min, 60% A → 90% A, 5.2 min, 90% A, 0.1 min 90% A → 60% A. Re-equilibration duration was 10 min between individual runs. A series of BA ethanol standard solutions with concentrations of 5~80 μg mL^{-1} were configured. Using concentration (C, μg mL^{-1}) as x-coordinate and integrated peak area (Y) as y-coordinate. The equation for calibration curve of BA is Y= 6705.24 C-14037.09, R^2 = 0.9997, indicating good linearity from 5 to 80 μg mL^{-1}. Results of the average recovery was 99.60% (RSD = 0.45%, n = 5) and precision RSD was 0.32% (n = 5).

2.7 *In vitro* Cytotoxicity of BA/DT-β-CD Complex against Hela Cell

Viable HeLa cells were seeded in 96-well culture plates at a density of 4 × 103 cells in 0.2 mL of growth medium per well and allowed to attach for 24 h. The culture medium was replaced with BA/DT-β-CD complex water solution, BA water suspension and BA DMSO solution at different concentrations in four replicates followed by 72 h of incubation. After incubation, 20 μL of MTT solution at a concentration of 5 mg mL^{-1} was added to each well followed by the 4 h of incubation. MTT solution was then aspirated and 150 μL of DMSO was added to each well to dissolve the dark blue crystals thoroughly. The absorbance was measured at 490 nm using a microplate reader (Infinite 200 NanoQuant, Tecan, Austria). The relative growth rate (%) was calculated as (mean absorbance of the sample/mean absorbance of the control) × 100 %, considering the optical density of the control as 100%.

3. RESULTS AND DISCUSSION

3.1 IR Analysis

The IR spectra of β-CD, DT-β-CD, BA, complexes of BA/DT-β-CD, BA/TT-β-CD and BA/TP-β-CD are shown in Fig. 3. Because the spectra of DT-β-CD, TT-β-CD and TP-β-CD show no difference, in this study, the spectrum of DT-β-CD is used for representing the spectra of oligo(polylvinylamino) bridged bis(β-cyclodextrin)s. The prominent peak assignments are listed in Table 1. From Table 1, the encapsulation of BA does not cause significant shifts of the IR bands as compared with oligo(polylvinylamino) bridged bis(β-cyclodextrin)s, indicating no chemical bonds are created in the formed complex, only intermolecular forces or hydrogen bonding interaction exists between the acid and DT-β-CD. However, some differences appear. The wavenumber for BA observed at 1687 cm^{-1} (Fig. 3c), which corresponds to the stretching vibration of association acid ν (C=O), is redshifted to 1625 cm^{-1} (Fig. 3d, 3e) and overlaps with the bending vibration of hydroxyl groups δ (O-H) for DT-β-CD (Fig. 3b). This amount of redshift is attributed to the conjugation effect for the nearby N atom which belongs to the host molecule of oligo(polylvinylamino) bridged bis(β-cyclodextrin)s.

Therefore, it is an evidence that the hydrogen bond exists between the secondary amine from the tethers of oligo(polyvinylamino) bridged bis(β-cyclodextrin) and the carboxyl group from the association acid of BA. Furthermore, as illustrated in Fig. 3f, 3e and 3d, the band intensity at 1625 cm^{-1} gradually increases in sequence. In fact, this band at 1625 cm^{-1} for BA/TP-β-CD complex is distinct weakening as compared with the complexes of BA/DT-β-CD and BA/TT-β-CD. This phenomenon indicates that, TP-β-CD and TT-β-CD, DT-β-CD have an enhanced molecular binding ability towards the molecule BA in turn. In other words, the rigid spacer of the tethers plays a significant role during the encapsulation process.

In addition, the band at 1401 cm^{-1} (Fig. 3d and 3e), which is attributed to the bending vibration of methyl group from BA, is observed in IR spectra of the formation complex. Furthermore, the O-H stretching band at 3394 cm^{-1} (Fig. 3d) decreases by 10 cm^{-1} as compared with that of DT-β-CD (Fig. 3b), besides, the band also becomes broader. This phenomenon indicates a change of hydrogen bonds structure, which is probably associated with a reorganization of the intramolecular hydrogen bonds formed between -OH and -NH- groups of DT-β-CD in the presence of BA. Similar results have also been found in the spectra of TT-β-CD and TP-β-CD.

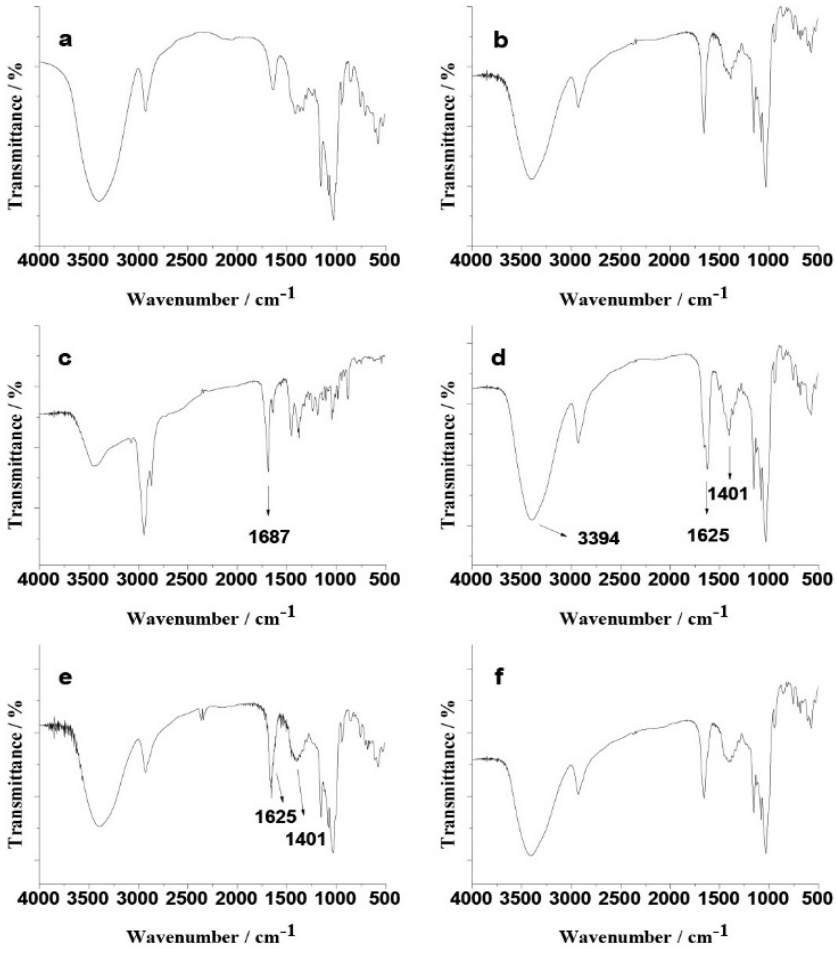

Fig. 3. IR spectra of (a) β-CD; (b) DT-β-CD; (c) BA; (d) BA/DT-β-CD complex; (e) BA/TT-β-CD complex; (f) BA/TP-β-CD complex

Table 1. The wavenumbers of BA, β-CD, DT-β-CD and BA/DT-β-CD

Chemical functional groups and vibration mode	Wavenumber / cm^{-1}			
	BA	β-CD	DT-β-CD	BA/DT-β-CD
Hydroxyl or carboxyl ν (O-H)	3448	3397	3404	3394
ν (C=C→H)	3072	-	-	-
Methyl and methylene ν (C-H)	2942, 2869	2928	2927	2928
Associated carboxyl ν (C=O)	1687	-	-	-
Vinyl ν (C=C)	1641	-	-	-
Methylene δ (C-H) and Methyl δ (C-H)	1452	1413	1437	1457
Methyl δ (C-H)	1376	1368	1388	1401
ν (C-O)	1237,1189	1157,1079, 1028	1155, 1080, 1031	1155, 1080, 1032
δ (O-H) or carboxyl ν (C=O) of carboxylate	overlapped	1640	1655	1654, 1625

3.2 SEM Analysis

The surface morphology of powders derived from β-CD, DT-β-CD, BA, complexes of BA/DT-β-CD, BA/TT-β-CD and BA/TP-β-CD, as assessed by SEM, is provided in Fig. 4. Because of the similar surface morphology of DT-β-CD, TT-β-CD and TP-β-CD, in this study, the SEM picture of DT-β-CD is used for representing the pictures of all the three oligo(polylvinylamino) bridged bis(β-cyclodextrin)s.

Firstly, comparing the picture of DT-β-CD with that of β-CD (Fig. 4a), some crosslinking can be noticed in the picture of DT-β-CD (Fig. 4b) for the homogeneous plate-like structure crystal morphology, while β-CD demonstrates a petal-style structure crystal (Fig. 4a).

Secondly, comparing the pictures of BA/DT-β-CD (Fig. 4d) and BA/TT-β-CD (Fig. 4e) with those of DT-β-CD (Fig. 4b) and BA (Fig. 4c), these pictures of complexes are structurally distinct from the isolated components, those being the unmanipulated BA and DT-β-CD. BA (Fig. 4c) appears as acicular crystal structure, while DT-β-CD (Fig. 4b) appears as irregularly shaped crystal particles. However, the BA/DT-β-CD and BA/TT-β-CD complexes (Fig. 4d and 4e) appear as a compact and homogeneous plate-like structure crystal and are quite different from BA and DT-β-CD in size and shape, which confirms the formation of the BA complexes. However, although the BA/TT-β-CD complex also appears as a compact and homogeneous plate-like structure crystal, the surface compactness and uniformity of the BA/TT-β-CD complex is inferior to that of the BA/DT-β-CD. That means BA is more suitable for the dimer cavity of DT-β-CD than TT-β-CD. Furthermore, the morphology of BA/TP-β-CD (Fig. 4f) complex has no difference from that of TP-β-CD (Fig. 4b), this result shows that the binding ability of TP-β-CD toward BA is the worst. In other words, the pseudo cavity of TP-β-CD is too large to well encapsulate the molecule of BA, BA can easily escape from the pseudo cavity of TP-β-CD and gives a worse binding ability than DT-β-CD and TT-β-CD.

3.3 ^{1}H NMR and Molecular Simulation Studies

After discussing the results of IR and SEM, three conclusions can be drawn. Firstly, oligo(polylvinylamino) bridged bis(β-cyclodextrin)s can encapsulate the molecule of BA. Secondly, the dimer cavity including the rigid spacer plays an important role during the encapsulation process. Thirdly, the binding ability of oligo(polylvinylamino) bridged bis(β-cyclodextrin)s toward BA decreases in the order of DT-β-CD, TT-β-CD and TP-β-CD. Therefore, to simplify the study, in the following work, DT-β-CD is used instead of the oligo(polylvinylamino) bridged bis(β-cyclodextrin)s for further results.

To investigate the encapsulation behavior, ^{1}H NMR and molecular simulation studies are used for demonstrating the interaction of the host molecule and guest molecule between DT-β-CD and BA. The molecular simulation result is shown in Fig. 5 and ^{1}H NMR results are shown in Fig. 6.

The objective of the ^{1}H NMR study is to demonstrate a possible displacement of DT-β-CD protons located inside the cavity (namely H3, H5 and H6) resulting from BA inclusion and interaction, consistent with the results of IR and SEM. However, in Fig. 6c, the ^{1}H NMR results show only weak displacements, presumably the molecule of BA is shallowly embedded into the cavity of DT-β-CD [55]. This experiment result can be verified by the molecular simulation result (Fig. 5). In Fig. 5, for the molecule size and steric hindrance, BA molecule can not fully occupy both cavities. Therefore, this encapsulation behavior leads to weak displacements of the H3, H5 and H6 located inside the cavity.

Compared with Fig. 6a and Fig. 6b, in Fig. 6c, the ^{1}H NMR spectrum of BA/DT-β-CD complex clearly demonstrates the presence of the framework protons of the BA molecule. The methyl protons of BA display chemical shifts at δ 0.5-1.1 ppm, which are distinct from the DT-β-CD protons, provide direct evidence for the formation of the inclusion complex. Furthermore, after inclusion complexation with BA, the H-3 protons of DT-β-CD shifts 0.010 ppm and the H-5 protons of DT-β-CD shifts 0.013 ppm. Both H-3 and H-5 protons are located in the interior of the CD cavity, with H-3 protons near the wide side of cavity and H-5 protons near the narrow side. These results may indicate that BA should be included in the DT-β-CD cavity from the narrow side.

3.4 MS Analysis

The MS spectra of BA standard solution and BA extracting solution are shown in Fig. 7. The molecular ion peak and quasi-molecular ion peak [M-H]- of the BA are observed at m/z = 456.2 and 454.8 in BA standard solution. These two peaks are also observed in the BA extracting

solution. On one hand, the result suggests that the inclusion complex of BA/DT-β-CD contains BA. On the other hand, the extracting method for de-clathration by ethanol is feasible. However, using the two peaks as reference for quantitative BA is not stable, therefore, the establishment of standard curve with the pieces peak of 456.2 or 454.8 is not feasible.

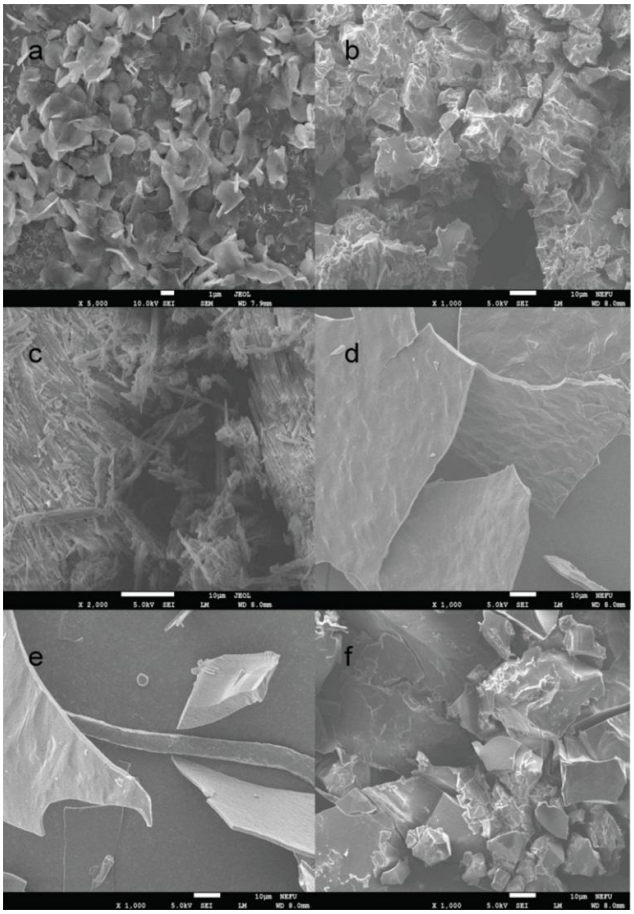

Fig. 4. SEM images of (a) β-CD; (b) DT-β-CD; (c) BA; (d) BA/DT-β-CD complex; (e) BA/TT-β-CD complex; (f) BA/TP-β-CD complex

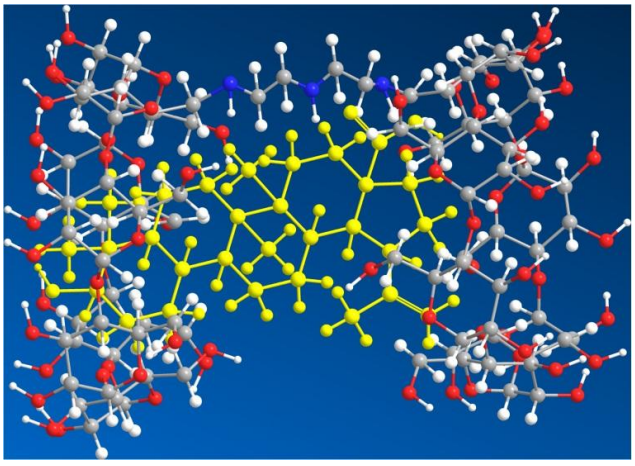

Fig. 5. A possible binging mode of BA/DT-β-CD complex

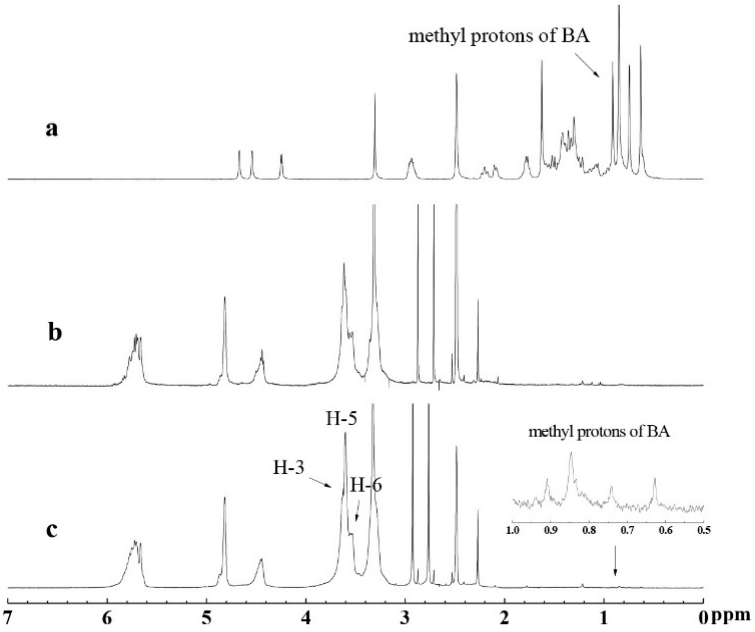

Fig. 6. ^{1}H NMR spectra of (a) BA; (b) DT-β-CD; (c) BA/DT-β-CD complex

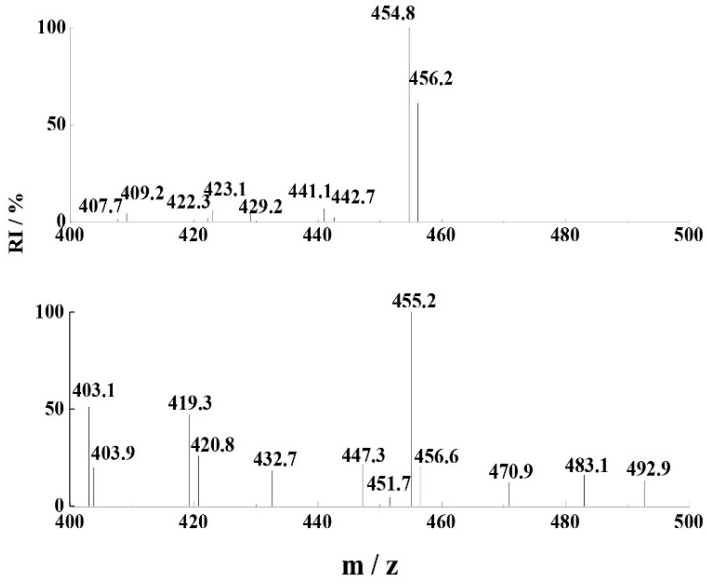

Fig. 7. (a) BA standard solution and (b) BA/DT-β-CD complex solution

3.5 Solubilization

BA water solubility, which is almost nil, is dramatically improved by inclusion complexation. The solubility of resulting complex of BA and oligo(polylvinylamino) bridged bis(β-cyclodextrin)s is assessed by the preparation of saturated complex solution. The BA content of the filtrate is tested by the UPLC (Fig. 8). The results show that the retention time of BA extracting solution (Fig. 8c, Fig. 8d) and BA standard solution (Fig. 8a) is the same, in 3.26 minutes, DT-β-CD (Fig. 8b) has no effect on the results. The first extraction amount of BA is 10.8 μg mL^{-1}, the second extraction amount of BA (Fig. 8d) is 5.0 μg mL^{-1}, and the third is not visible. Therefore, twice extraction by ethanol is enough for quantification.

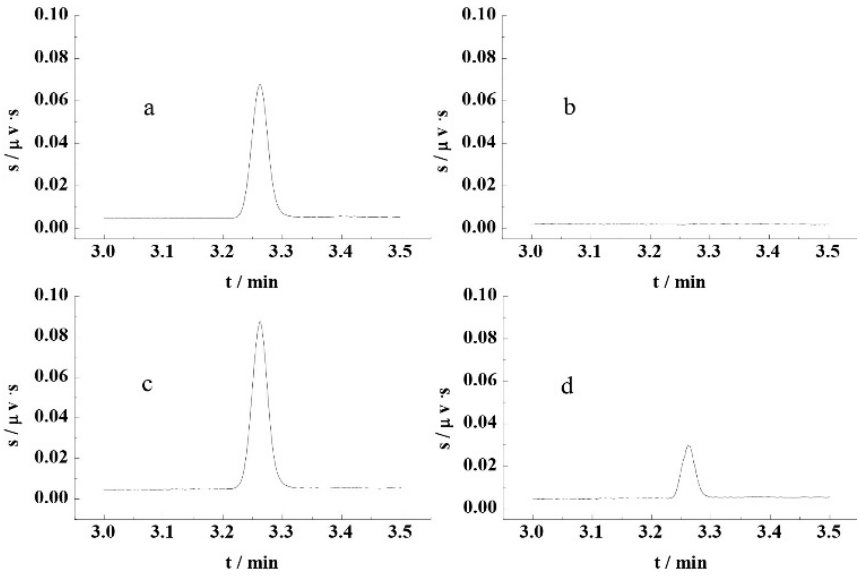

Fig. 8. UPLC chromatographs of (a) BA standard solution, (b) DT-β-CD ethanol solution, (c) BA/DT-β-CD complex solution after exacting once, (d) BA/DT-β-CD complex solution after exacting twice

Furthermore, the calculation results are shown in Table 2. In Table 2, it can be observed that the BA complex is easy to dissolve in water, 0.5 h of stirring is enough to reach the equilibrium. Compared with that of native BA (which is almost nil), the water solubility of this BA, is remarkably increased to approximately 310.97 μg mL^{-1} (680.89 μmol L^{-1}), 144.94 μg mL^{-1} (317.36 μmol L^{-1}) and 75.04 μg mL^{-1} (164.30 μmol L^{-1}) by the solubilizing effects of DT-β-CD, TT-β-CD and TP-β-CD (Fig. 9), respectively. The mass fractions of BA in BA/DT-β-CD, BA/TT-β-CD and BA/TP-β-CD complexes are 0.63 %, 0.29 % and 0.15 %, respectively.

Table 2. The solubilities of BA/DT-β-CD BA/TT-β-CD BA/TP-β-CD in the water solution

Stirring time	BA solubilities / μg mL^{-1}		
	BA/DT-β-CD	BA/TT-β-CD	BA/TP-β-CD
0.5 h	298.32	144.63	77.12
1.0 h	315.43	145.32	75.37
1.5 h	322.16	139.81	71.68
2.0 h	307.98	150.01	76.02
Average	310.97	144.94	75.04

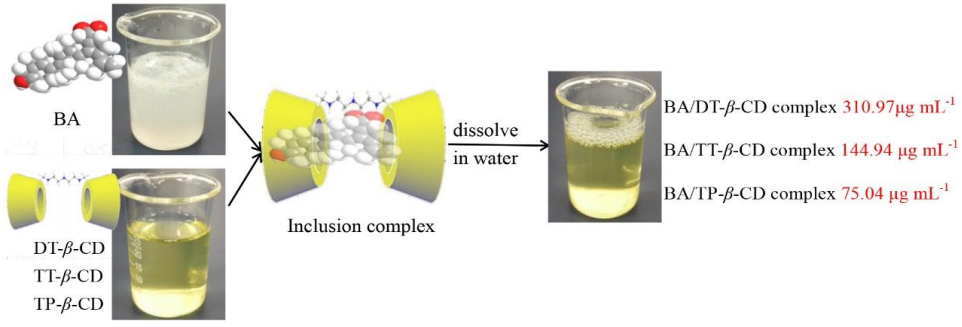

Fig. 9. Solubilization effect of BA encapsulated by DT-β-CD, TT-β-CD and TP-β-CD, respectively

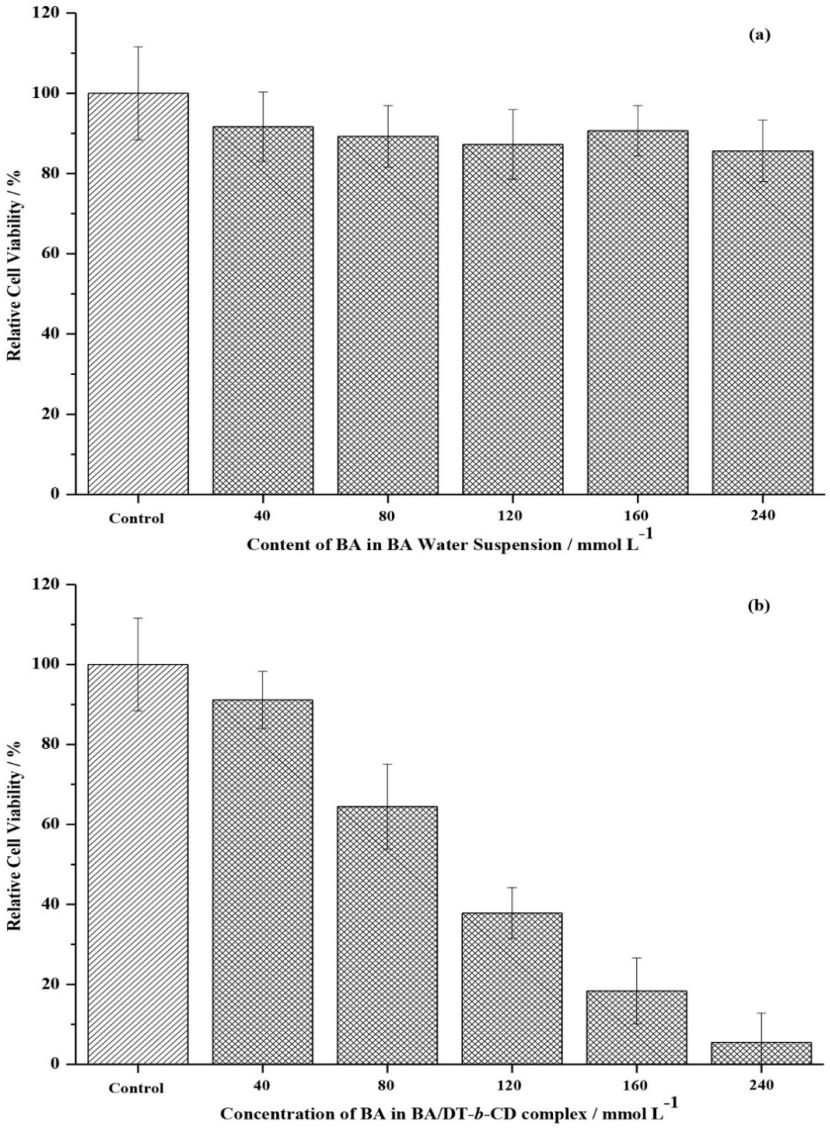

Fig. 10. The relative proliferation rates of HeLa cells exposed to different concentrations of (a) BA water suspension, (b) BA/DT-β-CD complex water solution

3.6 MTT Assay

To evaluate the cytotoxicity of BA/DT-β-CD complex water solution, an *in vitro* test against human cancer cell line HeLa was performed. The relative proliferation rates of HeLa cells were tested by exposing them to different concentrations of BA/DT-β-CD complex water solution and BA water suspension, respectively. The results are shown in Fig. 10. As shown in Fig. 10a, after 72 h incubation with sample solutions, BA water suspension does not give a significant inhibitory activity against HeLa cell, and cannot give a reliable IC50 value. However, in Fig. 10b, the IC_{50} value of BA in BA/DT-β-CD complex water solution is 103.89 ± 5.12 μmol L^{-1}, in consideration of the maximum of water solubility of BA/DT-β-CD complex (680.89 μmol L^{-1}), the property of easily dissolving in water can give BA/DT-β-CD complex a safer drug delivery way in the human HeLa cervical cancer therapies.

4. CONCLUSIONS

As an anticancer compound, the BA has a promising control effect on various kinds of cancers. Its water solubility remarkably increases

to several hundred μg mL^{-1} by the solubilizing effects of oligo(polylvinylamino) bridged bis(β-cyclodextrin)s. The results of IR, SEM, ^1H NMR and molecular simulation demonstrate that the dimer cavity including the rigid spacer can encapsulate the molecule of BA, the binding ability decreases in the order of DT-β-CD, TT-β-CD and TP-β-CD. The size of pseudo cavity composed by the dimer cavity and the rigid spacer plays an important role in the encapsulation process.

For quantification of BA in the BA complex, two times of extraction by ethanol is enough to meet the precision and accuracy requirements. The water solubility of complexes decreases in the order of DT-β-CD, TT-β-CD and TP-β-CD, consistent with the results of binding ability. The IC_{50} value of BA in BA/DT-β-CD complex water solution is 103.89 ± 5.12 μmol L^{-1}, in consideration of the maximum of water solubility of BA/DT-β-CD complex, BA/DT-β-CD complex can be an active agent for the treatment of human HeLa cervical cancer, and can give a safer drug delivery way and large dose application possibility in the human HeLa cervical cancer therapies.

ACKNOWLEDGEMENT

This study is supported by the National Natural Science Fund of China (NSFC, 51403030) and the Fundamental Research Funds for the Central Universities of China (2572017CB23). National Students' platform for innovation and entrepreneurship training program (201610225103) is also gratefully acknowledged for support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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