Preliminary communication

Synthesis of novel dendrimers having aspartate grafts and their ability to enhance the aqueous solubility of model drugs

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ABSTRACT

In this study, a series of aspartate based dendrimers with different cores were synthesized in a convergent approach and well characterized by NMR and MS techniques. The aqueous solubility of the model drugs (L-Histidine, Naproxen, Methotrexate) was measured in the presence of this kind of dendrimers at room temperature in PBS buffers at pH 6, 7 and 8. Results clearly confirmed that the solubility enhancement was due to presence of dendrimers at different pH compared to their corresponding aqueous solubility at different pH. The results indicated that the aspartate based dendrimers could be considered as an effective supplement of PAMAM dendrimers in solubility enhancement and drug delivery. The surface groups played an important role in dendrimer-mediated solubility enhancement.

1. Introduction

Dendrimers are a novel type of polymeric material that have highly branched structure and globular shape. The classic chemical architecture of dendrimers possesses a well-defined core, an interior region and a large number of end groups [1]. The physical characteristics of dendrimers, including their monodispersity, hydrophilicity, encapsulation ability, and large number of functionalizable peripheral groups, make these macromolecules ideal candidates for evaluation as drug delivery vehicles [2–5].

The family of dendrimers most investigated in drug delivery is the poly(amido amine) dendrimers (PAMAM). PAMAM dendrimers are biocompatible, non-immunogenic, water soluble and possess terminal modifiable amine functional groups for binding various targeting or guest molecules [6]. The high density of amino groups and internal cavities in PAMAM dendrimers is expected to have potential applications in enhancing the aqueous solubility of low

solubility drugs [7–9]. Generation 0.5 (G0.5) to generation 4 (G4) PAMAM dendrimers have been used to encapsulate and solubilize acidic drugs such as Nifedipine [10], Ibuprofen [11] and Indomethacin [12]. Further, PAMAM derivatives modified by –OH or –COOH groups were found to encapsulate small hydrophobic guest molecules successfully: Twyman et al. [13] converted the ester terminated PAMAM dendrimers to a more soluble hydroxyl surface by reacting with TRIS (Tris (hydroxymethyl)aminomethane). The –OH-terminated dendrimers were found to increase solubility by weak hydrogen bonding; Neofistou and Demadis [14] synthesized the PAMAM dendrimers with carboxy groups in peripheral and used these kinds of dendrimers as the solubility enhancer of SiO_2.

Recently, more dendritic molecules were investigated as solubility enhancers such as poly-lysine (PLS) [15], poly-propyleneimine (PPI) [16], and poly-citric acid dendrimers (PCA) [17]. Several previous reviews have covered the early work of the solubility enhancement with dendrimers. The possible mechanisms of the dendrimer-mediated solubility enhancement are based on a typical dendrimer's structure: (a) a central core with enough internal cavities is available to encapsulate the guest molecules; (b) branching units provide weak hydrogen bonding to increase solubility; (c) the surface groups are mainly responsible for the solubility enhancement by electrostatic interactions [18–20]. Some experts supposed that the hydrophilic surfaces and

hydrophobic interiors made the dendrimers 'static unimolecular micelles' and these unimolecular micelles were able to encapsulate a number of insoluble drug molecules [21].

In our previous work, prodrugs with aspartate based dendrimers were studied [22,23]. A comparative solubility enhancement profile was found when the aspartate based dendrimers were conjugated to an insoluble drug molecule and the water solubility was increased by the structures with more polar groups [23]. On the basis of the mechanisms above and the initial studies, we began to consider the aspartate based dendrimers could be used as a solubility enhancer just like the PAMAM dendrimers. One hand, as the same of PAMAM dendrimers, this kind of structures consisted of amido linkage and the carbon–carbon bond length was large enough, which created a similar internal microenvironment with similar polarity, the other hand, opposed to PAMAM dendrimers and other dendrimers, the surface groups of this kind of dendrimers were acidic groups: –COOH instead of the alkaline groups: –NH$_2$. This feature might display new properties in the acid/base mediated solubility enhancement of some acidic or alkaline drug molecules.

Anticipated potential of aspartate based dendrimers as solubilizing agents, their similar hydrophobic microenvironment and different surface groups compared to PAMAM dendrimers and paucity of reports proving these kinds of dendrimers prompted us to design a comprehensive study on aspartate based dendrimers mediated solubilization. It was reported that solubilization potential of dendrimers increased with molecular size. Therefore we designed to utilize different cores to construct aspartate based dendrimers. The aim of the present work was (1) to synthesize Generation 1 aspartate based Dendron (G1-Asp-Dendron) and attach them to different dendritic cores to form a well-defined highly branched architecture; (2) to study the potential of aspartate based dendrimers as a solubility enhancer of the model drugs; (3) to study the effect of pH, concentration, numbers of surface functional group on interactions between the dendrimers and the model drugs. Naproxen, Methotrexate and L-Histidine were used for model drugs because they were clinically relevant agents and not freely soluble in water at room temperature, furthermore, they were representatives of different acid/base drugs: weakly acidic Naproxen, –COOH functional group; weakly acidic and weakly basic Methotrexate, –COOH and –NH$_2$ functional group; weakly basic L-Histidine, imidazole ring sidechain.

2. Chemistry

All the dendritic cores, compounds **1–4** (Fig. 1), were synthesized by literature methods [24,25]. The synthesis involved two consecutive chain-forming reactions, the exhaustive Michael addition reaction and the functional group changing reactions.
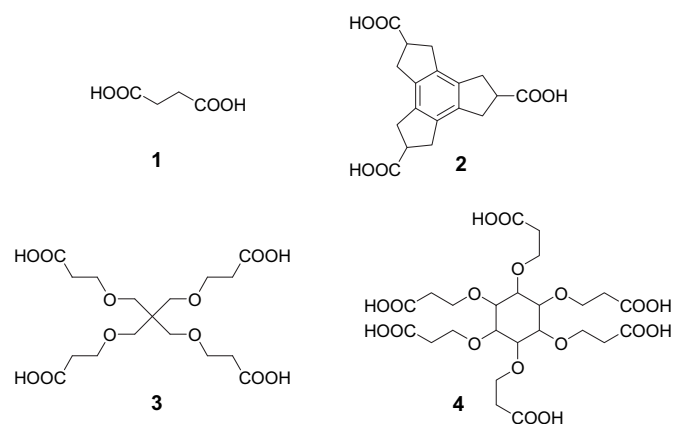

Fig. 1. The chemical structure of dendritic cores.

The G1-Asp-Dendron (Compound **5**) was prepared by the same method in our previous work [23]. 2-(tert-Butoxycarbonyl)succinic acid (Boc-Asp) and Dibenzyl 2-aminosuccinate (NH$_2$-Asp-OBzl$_2$) were connected by amidation reaction using DCC/HOBt (Dicyclohexylcarbodiimide/Hydroxybenzotriazole). The protected group (Boc–) was removed in TFA/DCM (Trifluoroacetic acid/Dichloromethane). The synthetic procedure was outlined in Scheme 1.
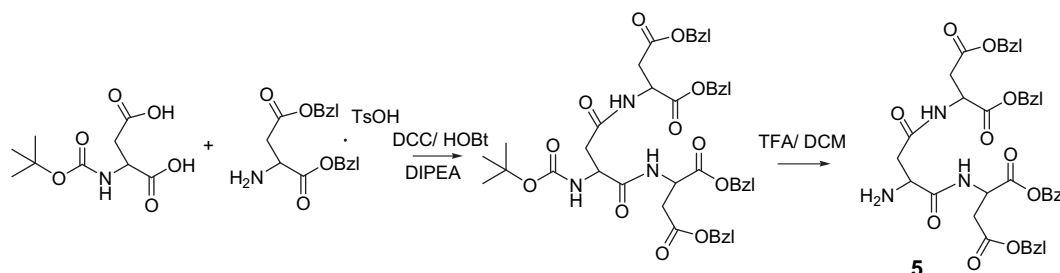
In the synthesis of the target dendrimers (**T1**–**T4**), for the large size of the reactant, using DCC/HOBt did not give the product in acceptable yields. Only a solid phase peptide coupling reagent HBTU/DIPEA (o-Benzotriazol-1-yl-tetramethyluronium hexafluorophosphate/N,N-Diisopropylethylamine) afforded the products in good yields. The synthetic procedure was outlined in Scheme 2.

3. Results and discussion

3.1. Structural characterization of the synthetic peptide dendrimers

The characteristic data of the synthetic dendrimers was outlined in Table 1. The high degree of symmetry in these dendrimers enabled facile confirmation of both structure and purity by NMR techniques. For example, in the ^{1}H NMR spectrum of dendrimer **T3** the core protons observed the resonance signals at 2.37 (brs), 3.2 (s), and 3.55 (brs) ppm were clearly distinguishable from the resonances arising from the wedges (G1-Asp-Dendron, Compound **5**) at 2.65 (m) and 4.72 (m) ppm. Integration of the respective areas of the core protons and β-methine protons of G1-Asp-Dendron confirmed the complete coupling of the central core and peptides.

Furthermore, the structures of these dendrimers were further verified by ESI MS electrospray ionization mass spectrometry and MALDI MS matrix-assisted laser desorption/ionization mass spectrometry [32,33]. All of the ESI MS spectra displayed a very

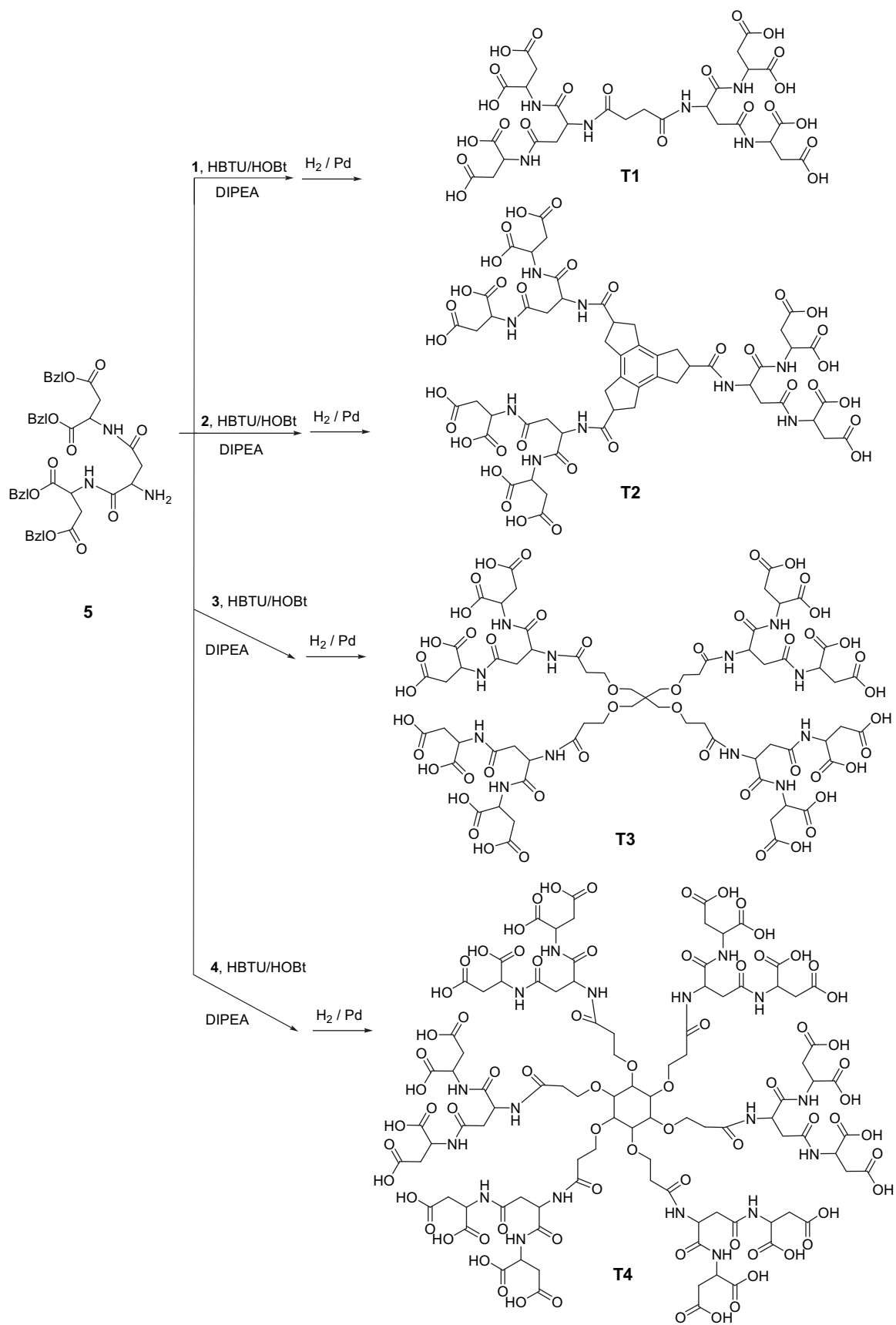**Scheme 1.** The synthesis of G1-Asp-Dendron.

Scheme 2. The synthesis of target dendritic molecules.

Table 1
The characteristic data of the synthetic dendrimers.

Compound	Molecular formula	Molecular weight	Number of internal amide groups	Number of terminal carboxyl groups
T1	$C_{28}H_{36}N_6O_{22}$	808.61	6	8
T2	$C_{54}H_{63}N_9O_{33}$	1365.12	9	12
T3	$C_{65}H_{88}N_{12}O_{48}$	1805.45	12	16
T4	$C_{96}H_{126}N_{18}O_{72}$	2684.11	18	24

prominent peak corresponding to the dendrimers complexed with protons or sodium cation. The results of MALDI MS were less satisfactory, but a molecular ion peak could still be easily found. These differences between ESI and MALDI, in particular, depended on the extent of dendrimer–matrix reactions [34]. Moreover, elemental analysis was also in good agreement with those of the signed structures.

3.2. Characterization of Naproxen–dendrimer solubility profiles

Naproxen is one of the acidic non-steroidal anti-inflammatory drugs (NSAIDs) with a functional group –COOH ($pK_a = 4.2$), so its solubility in water is pH-dependent [11]. Because the acidity of naproxen, the inherent solubility was 1.18 mg/ml, pH = 6; 2.24 mg/ml, pH = 7; 24.45 mg/ml, pH = 8. We measured the solubility of naproxen in the presence of **T1**–**T4** dendrimers at different acid/base conditions pH 6, 7, and 8. The solubility profiles were shown in Fig. 2.

It was observed that the **T1** dendrimer did not nearly increase the aqueous solubility of Naproxen at pH 6 and 7, but at pH 8, there was a little enhancement: from 24.45 up to 30.78 mg/ml. When experiments mentioned above are excluded, the other dendrimers **T2**–**T4** significantly increased the aqueous solubility of Naproxen at all three pH values. In the presence of **T2**–**T4** dendrimer at all pH values, the solubility of Naproxen increased in an approximately linear manner with an increase in dendrimer concentration and the increased solubility was highest at pH 8, less at pH 7 and least at pH 6. It is clear that the solubility of Naproxen was affected by the increasing molecular weight and surface functional groups of the dendrimers **T1**–**T4**: take **T4** dendrimer for example, after interactions with the dendrimer at concentration of 0.01 mol/L, Naproxen's solubility increased from 1.18 to 2.51 mg/ml at pH 6, from 2.24 to 4.66 mg/ml at pH 7, from 24.45 to 81.92 mg/ml at pH 8.

3.3. Characterization of Methotrexate–dendrimer solubility profiles

Methotrexate is one of the prototype folate antagonist cytotoxic drugs used in non-malignancy and non-malignant diseases. The molecule is made up of a heterocyclic portion (a 2,4-diaminosubstituted pterine ring) linked to an aminobenzoyl portion, which is, in turn, an amide bonded to a glutamic acid [27]. Hence, MTX is a polyelectrolyte carrying two carboxyl groups, with pK_a's of 3.36 (α-carboxyl) and 4.70 (γ-carboxyl), and a number of potentially protonated nitrogen functions, the most basic of which, presumably, is the guanidinic N-1 on the pterine ring (pK_a 5.71) unit to an aminobenzoyl. The inherent solubility of methotrexate was 0.76 mg/ml, pH = 6; 5.75 mg/ml, pH = 7; 36.45 mg/ml, pH = 8. The Methotrexate–dendrimer solubility profiles were shown in Fig. 3.

It was observed that the solubility enhancement was similar to Naproxen, but the extent was less: take **T4** dendrimer for example, after interacting with the dendrimers at concentration of 0.01 mol/L, Methotrexate's solubility increased from 0.76 to 1.04 mg/ml at pH 6, from 5.75 to 7.62 mg/ml at pH 7, from 36.45 to 51.46 mg/ml at pH 8.

At higher concentrations of the dendrimers, the solubility of Methotrexate was slightly lower than predicted. This might be because of precipitation of an insoluble complex at high
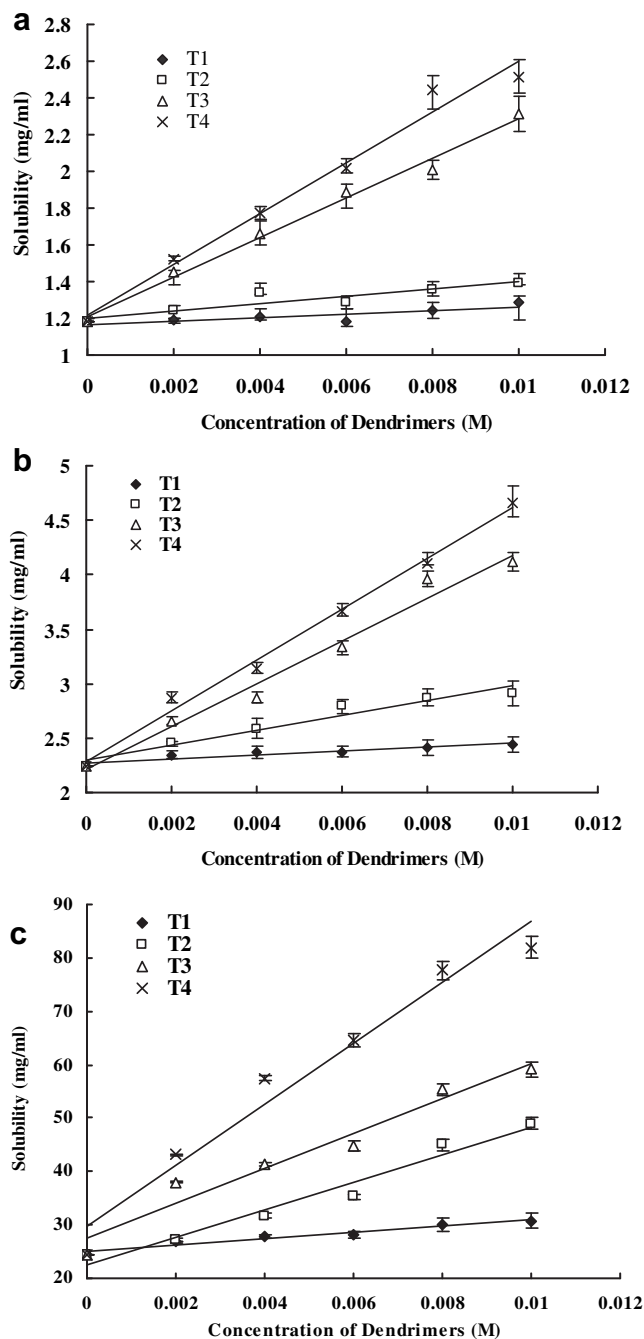
Fig. 2. Solubility profiles of Naproxen in the presence of increasing concentrations of aspartate based dendrimers in different pH: (a) pH = 6; (b) pH = 7; (c) pH = 8.

concentrations of **T1**–**T4** dendrimers. In fact, we observed yellow precipitation at higher concentrations in our experiments.

3.4. Characterization of L-Histidine–dendrimer solubility profiles

L-Histidine is one of the 20 standard amino acids presenting in proteins and it is considered as an essential amino acid in human body nutritionally. The imidazole side-chain of Histidine has a pK_a of approximately 6, and overall, the amino acid has a pK_a of 7.6. So Histidine is a positively charged weakly basic amino acid under physiological environment [28]. The inherent solubility of histidine was 98.84 mg/ml, pH = 6; 40.91 mg/ml, pH = 7; 97.33 mg/ml,
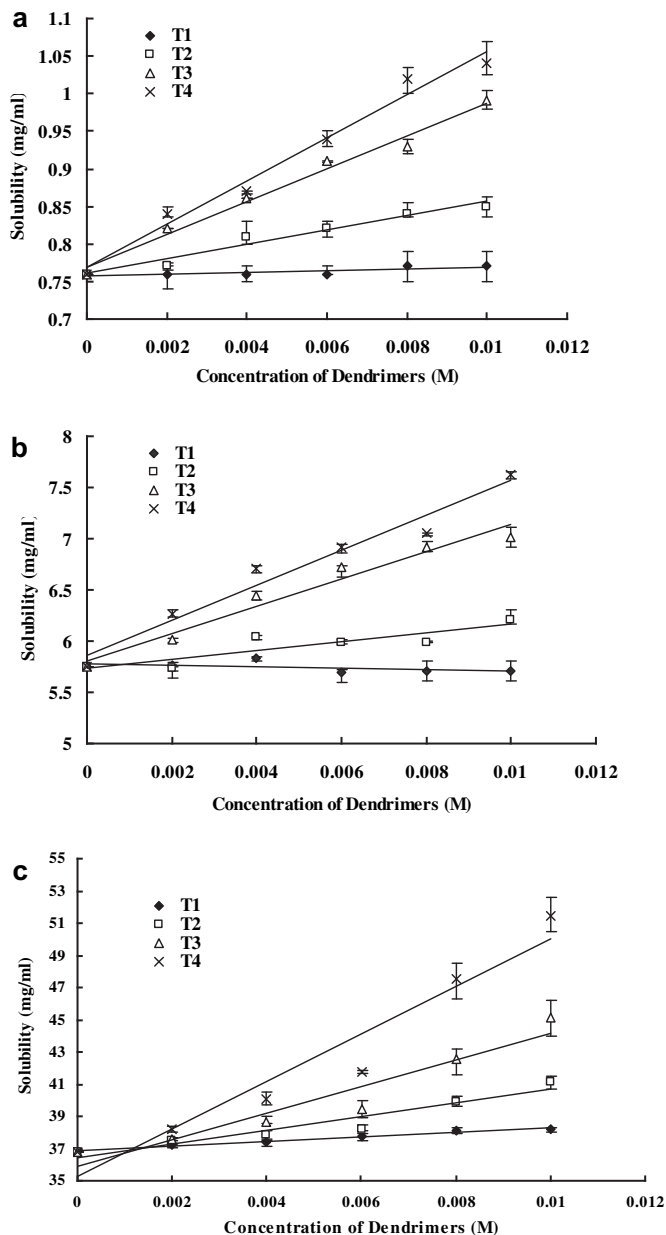
Fig. 3. Solubility profiles of MTX in the presence of increasing concentrations of aspartate based dendrimers in different pH: (a) pH = 6; (b) pH = 7; (c) pH = 8.

Fig. 4. Solubility profiles of L-Histidine in the presence of increasing concentrations of aspartate based dendrimers in different pH: (a) pH = 6; (b) pH = 7; (c) pH = 8.

pH = 8. The L-Histidine–dendrimer solubility profiles were shown in Fig. 4.

It was observed that the dendrimer-mediated L-Histidine solubility enhancement was very obvious: the solubility increased from 98.84 to 361.2 mg/ml at pH 6, from 40.91 to 97.33 mg/ml at pH 7, from 31.75 to 99.12 mg/ml at pH 8.

3.5. Possible mechanism of interactions between aspartate based dendrimers and model drugs

For the synthesized aspartate based dendrimers (**T1**–**T4**), the order of dendrimer-mediate solubility enhancement was **T4** > **T3** > **T2** > **T1**. Compound **T4** exhibited the most solubility enhancement in all model drugs and at all pH conditions, but compound **T1** exhibited almost no increased solubility in some conditions. It was presumably due to the increase in the size of dendrimer molecules and the internal architecture of dendrimers
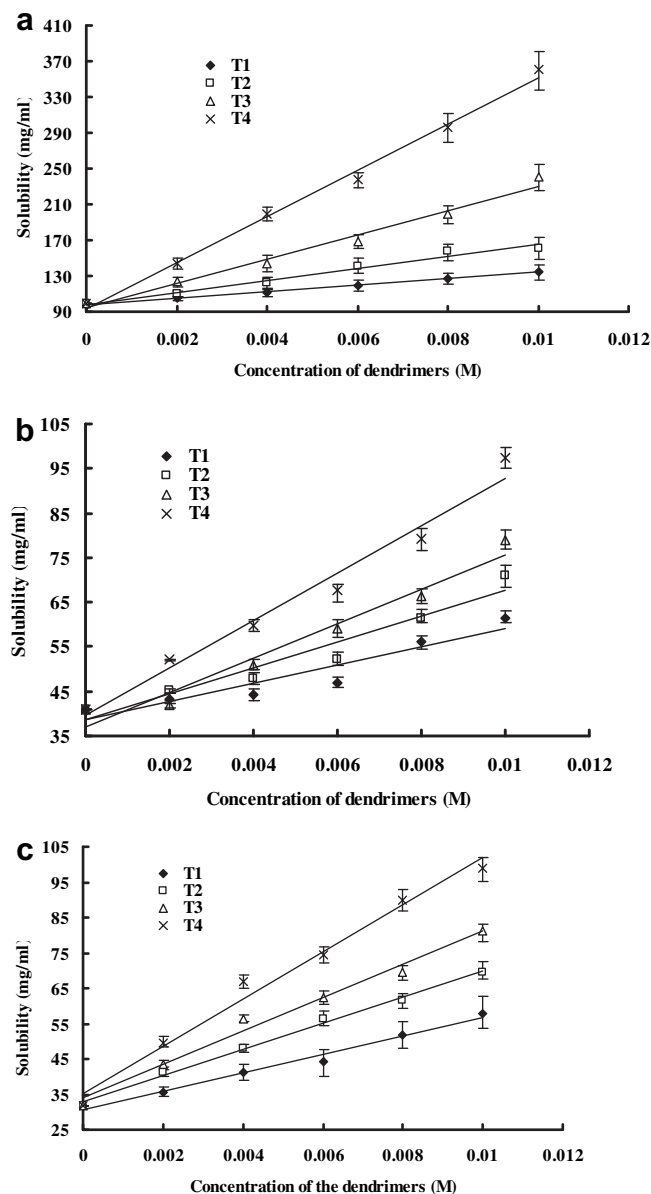
was well suited for the host–guest interactions and encapsulation of the biological model drug molecules. More importantly, the increased surface carboxy groups in periphery and tertiary amines in internal were available to interact with model drug molecules [29]. This special structure of dendrimer molecules could act as hydrogen bond donors and acceptors which could interact with the atoms of model drug molecules by hydrogen bond formation. Furthermore, the dendrimer structure had carboxyl groups on the surface which established electrostatic interactions with different parts of the drug molecules. So these total interactions were due in partly to hydrogen and partly to ionic bonds.

For the different model drugs, the order of dendrimer-mediated solubility enhancement was L-Histidine > Naproxen > Methotrexate. The molecular size of L-Histidine was small and it was easy to form a dendrimer-complex through a simple encapsulation. Further, L-Histidine had an imidazole ring side-chain which could attribute an electrostatic interaction between anionic dendrimers and the basic groups of L-Histidine. Naproxen possessed a carboxyl groups and

became considerably polar (negative ion) at the test pH which could reduce the electrostatic interactions. The solubility enhancement at the test pH could be ascribed to both hydrophobic interaction and hydrogen bonding between deprotonated/unionized fractions of dendrimer/Naproxen or though a simple encapsulation. Methotrexate exhibited lower level of solubility enhancement most probably due to the size of drug molecules preventing an efficient access to the internal cavities (pores) of the dendrimers [30].

As explained earlier, electrostatic interaction between hydrophobic drugs and peripheral groups of dendrimers is a major mechanism responsible for solubility enhancement. Protonation behaviour and pH of dendrimers affect the solubilizing power of dendrimers. As the pK_a of the –COOH of the aspartic acid was about 4.2, being weakly acid, the surface groups of the dendrimers became more ionized/protonated with the pH increasing. Cationic hydrophobic drugs might rarely be entrapped inside these dendrimers but the electrostatic interaction would be favoured for the anionic species and the peripheric negative charges of the anionic dendrimers would attribute the electrostatic interactions with the drug molecules. So, a pH-dependent solubility enhancement was observed. The solubility was highest at pH 8 and least at pH 6. This result further demonstrated that the surface groups played an important role in dendrimer-mediated solubility enhancement [31]. It can also be concluded that mechanism of solubilization largely depends on protonated/deprotonated state of dendrimer.

4. Conclusion

We had prepared a range of aspartate based dendrimers bearing two to six G1-Asp-Dendron sequences in periphery. The synthetic dendrimers had the potential to significantly enhance the solubility of poorly water soluble drugs, such as L-Histidine, Naproxen and Methotrexate. The increase in drug solubility in aqueous solution depended on the concentration and molecular size of the dendrimer, the numbers of surface functional groups and the pH of the medium. All observations were evidence that the enhancement of drug solubility was owing to electrostatic interactions and hydrogen bonding between anionic dendrimers and functional groups of the model drugs.

From the initial results, we found the aspartate based dendrimer could be considered as an effective supplement of PAMAM dendrimers in drug encapsulation and drug solubilization. We are continuing exploring higher generation of dendritic Asp peptides, developing controlled release drug delivery systems containing the drug trapped inside the dendrimers.

5. Experimental protocols

5.1. General

The reactions requiring anhydrous conditions were performed in an Ar or N_2 atmosphere. Thin layer chromatography (TLC): silica gel plates GF254; Compounds were visualized by irradiation with UV light and/or by treatment with a solution of phosphomolybdic acid (20 wt.% in ethanol) followed by heating. ^{1}H NMR analysis was performed by the INOVA VARIAN 400 MHz spectrometer using $CDCl_3$ or D_2O as a solvent at room temperature. Elemental analysis was performed by Atlantic Microlab, Atlanta, GA, USA. ESI mass spectrum was recorded with Agilent 1946B ESI-MS instrument, MALDI TOF MS was recorded with Bruker BIFLEX III (matrix was sinapic acid SA) and the UV analysis was carried out by the Cary 100 UV–Vis spectrophotometer. Naproxen and Methotrexate were obtained from Henrui Medicine Co., Ltd. (Jiangsu, China). L-Histidine was purchased from GL Biochem Ltd. (Shanghai, China). The other chemicals and solvents were A.R. grade and purified by

standard techniques. For solubility studies, water used to prepare solutions was two-distilled water.

5.2. Synthesis of the compound T1

A sample of succinic acid (0.12 g, 1.0 mmol) dissolved in DCM (20 ml) was cooled in an ice-water bath and the unprotected G1-Asp-Dendron 5 (1.50 g, 2.1 mmol) was then added to the reaction mixture, followed by DIPEA (0.23 g, 2.1 mmol), HBTU (0.80 g, 2.1 mmol) and HOBt (0.27 g, 2.1 mmol). The reaction mixture was stirred for 3 days. After evaporated under reduced pressure, the residue was taken up in ethyl acetate (25 ml) and washed with 1 M HCl (10 ml), brine (10 ml), 1 M $NaHCO_3$ (10 ml). The crude product was purified by silica gel column chromatography using DCM–methanol (50:1, v/v) as an eluent to yield a white solid (0.94 g) Yield: 61%.

The mixture of the obtained solid (0.94 g) and 10% Pd/C (200 mg) in methanol was stirred at the room temperature under a H_2 atmosphere. After 24 h the mixture passed through a membrane filter to remove the catalyst, then evaporated under reduced pressure. The residue was triturated with pure ethyl ether to afford T1 as a white powder (0.45 g). Yield: 91%.

5.2.1. Data

^{1}H NMR (400 MHz, D_2O): 2.51 (s, 4H, SA-CH_2), 2.78–2.96 (m, 12H, Asp-βCH_2), 4.79–4.96 (m, 6H, Aspα-CH). ESI MS (m/z): calcd. for 808.6. obsd. 831.2 ([M + Na]$^+$). MALDI TOF MS (m/z): calcd. for 808.6. obsd. 810.3 ([M + 2H]$^+$). Anal. calcd. for $C_{28}H_{36}N_6O_{22}$: C, 41.59, H, 4.49, N, 10.39. found: C, 41.77, H, 4.33, N, 10.37.

5.3. Synthesis of the compound T2

According to the same procedure of preparation T1, the target molecule T2 (0.36 g, white powder) could be prepared form 2 (0.2 g, 0.6 mmol). Yield: 44% in two steps.

5.3.1. Data

^{1}H NMR (400 MHz, D_2O): 2.56–2.96 (m, 18H, Asp-βCH_2), 3.02–3.30 (m, 12H, Core-CH_2), 4.78–4.92 (m, 9H, Aspα-CH). ESI MS (m/z): calcd. for 1366.1. obsd. 1388.8 ([M + Na]$^+$). MALDI TOF MS (m/z): calcd. for 1366.1. obsd. 1387.7 ([M + Na]$^+$). Anal. calcd. for $C_{54}H_{63}N_9O_{33}$: C, 47.48, H, 4.65, N, 9.23. found: C, 47.37, H, 4.79, N, 9.31.

5.4. Synthesis of the compound T3

According to the same procedure of preparation T1, the target molecule T3 (0.41 g, white powder) could be prepared form 3 (0.2 g, 0.4 mmol). Yield: 49% in two steps.

5.4.1. Data

^{1}H NMR (400 MHz, D_2O): 2.37–2.44 (brs, 8H, Core-$COCH_2$), 2.65–2.94 (m, 24H, Asp-βCH_2), 3.20 (s, 8H, Core-CCH_2), 3.55–3.60 (brs, 8H, Core-OCH_2), 4.72–4.91 (m, 12H, Aspα-CH). ESI MS (m/z): calcd. for 1805.5 obsd. 1806.8 ([M + H]$^+$). MALDI TOF MS (m/z): calcd. for 1805.5. obsd. 1828.7 ([M + Na]$^+$). Anal. calcd. for $C_{65}H_{88}N_{12}O_{48}$: C, 43.24, H, 4.91, N, 9.31. found: C, 43.31, H, 4.98, N, 9.18.

5.5. Synthesis of the compound T4

According to the same procedure of preparation T1, the target molecule T4 (0.29 g, white powder) could be prepared form 4 (0.2 g, 0.3 mmol). Yield: 33% in two steps.

5.5.1. Data

^{1}H NMR (400 MHz, D_2O): 2.42–2.51 (brs, 12H, Core-$COCH_2$), 2.66–2.91 (m, 36H, Asp-βCH_2), 3.37–3.42 (brs, 6H, Core-cycloH), 3.62–3.68 (brs, 12H, Core-OCH_2). 4.71–4.96 (m, 18H, Aspα-CH). ESI MS (m/z): calcd. for 2684.1 obsd. 2706.9 ($[M + Na]^+$). MALDI TOF MS (m/z): calcd. for 2684.1 obsd. 2709.1 ($[M + Na]^+$). Anal. calcd. for $C_{96}H_{126}N_{18}O_{72}$: C, 42.96, H, 4.73, N, 9.39. found: C, 43.14, H, 4.87, N, 9.17.

5.6. Solubility experiments

The aqueous solubility of model drugs was determined at pH 6, 7 and 8 in phosphate buffers (0.05 M NaH_2PO_4). The method used for sample preparation was similar to each system [26], i.e. excess drugs were added to 5 ml of each test solution containing increasing amounts of the dendrimers to ensure the drug solution reaching saturation and the solutions were mechanically shaken for 10 min at 37 °C. Then the solutions were centrifuged at 5000 rpm for 1 min and the absorbance of the drug molecules was tested at the characteristic wavelengths (Naproxen: 273 nm, Methotrexate: 306 nm, L-Histidine: 230 nm).

Acknowledgement

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