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Inclusion complexes of isoflavones with two commercially available dendrimers: Solubility, stability, structures, release behaviors, cytotoxicity, and anti-oxidant activities

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ABSTRACT

We prepared and characterized the inclusion complexes of daidzein with poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) dendrimers. Aqueous solubility of daidzein was significantly enhanced by both PAMAM and PPI (186- and 650-fold at 0.36 mM, respectively). Daidzein in G3 PAMAM solution is more stable than that in G4 PPI. NMR studies reveal the encapsulation of daidzein within the interior cavities of PPI through hydrophobic interactions. Daidzein exhibits a slower release behavior from PPI than that from PAMAM. PPI/daidzein complex is much more toxic than PAMAM/daidzein complex on several cell lines. PAMAM/daidzein complexes showed similar protective effect on oxidative stress-induced cytotoxicity as compared to free daidzein. These results suggest that the inclusion of daidzein with dendrimer can effectively improve the solubility, prolong the delivery, and maintain the anti-oxidant activity of daidzein. This research provides new insights into dendrimer-based drug delivery systems and will be helpful for the design of novel dendrimer/drug formulations.

1. Introduction

Isoflavones are a class of natural phenolic compounds abundant in soybeans and other legumes (Reiter et al., 2009). The chemical structures of isoflavones are similar to that of human estrogen (Warri et al., 2008). As a result, isoflavones can be used to block estrogen from binding to its receptors on cell membrane, thus have several favorable health benefits such as reducing the risk of estrogen-dependent cancers including breast, ovarian, and endometrial cancers (Jiang et al., 2010). In addition to their estrogenic properties, isoflavones were reported to have antioxidant properties by enhancing the activities of catalase, superoxide dismutase and glutathione reductase (Barbosa et al., 2011). They can also be used to prevent osteoporosis (Byun and Lee, 2010). Therefore, isoflavones have attracted increasing attentions in clinical applications (Reiter et al., 2009; Ward and Kuhnle, 2010; Zhang et al., 2011).

However, isoflavones have poor lipophilicity and hydrophilicity, which brings a major challenge in pharmaceutical applications of these compounds (Zhang et al., 2011). Firstly, low aqueous solubility prevents the administration of isoflavones by injections and other liquid forms. Secondly, oral administration of these isoflavones with extremely low aqueous solubility faces low bioavailability problems. Thirdly, isoflavones are rapidly metabolized in the livers and intestines, leading to low absorption of these bioactive compounds (Lee et al., 2007). To enhance solubility and bioavailability of isoflavones, cyclodextrins and their derivates (Cannava et al., 2010; Lee et al., 2007; Stancanelli et al., 2007; Xavier et al., 2010), cyclodextrin/hydrophilic polymer mixtures (Borghetti et al., 2011), phospholipids (Gao et al., 2008; Zhang et al., 2011), surfactants (Whaley et al., 2006), chitosan microspheres (Ge et al., 2007), poly(L-lactide) (Sojitra et al., 2010), hyperbranched polyester (Zou et al., 2005), and emulsions (Shen et al., 2010) were used as potential carriers. The solubility and bioavailability of isoflavones were improved in these studies, but the increased solubility of isoflavones still cannot meet the clinical need since the solubility was only increased by an order of magnitude in the presence of cyclodextrins (Lee et al., 2007; Stancanelli et al., 2007; Xavier et al., 2010). Though the assembled aggregates such as phospholipid nanospheres can improve the isoflavone solubility to a much higher extent, these supramolecular capsules are not stable and may disassemble into monomers followed by rapid release of encapsulated drugs when they were administrated (Gao et al., 2008; Zhang et al., 2011). Thus, there is an urgent demand for the development of

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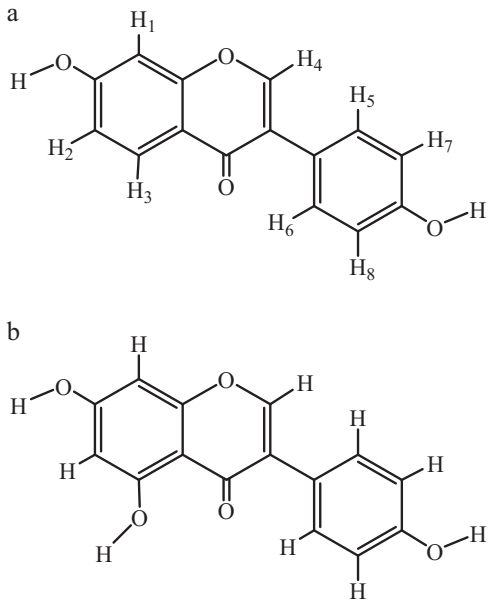


Scheme 1. Molecular structures of isoflavones (a) daidzein and (b) genistein. Daidzein structures are shown with proton labeling.

vehicles for isoflavones with stable structures and high drug payload.

Dendrimers are a class of nanoscale macromolecules with well-defined numbers of surface functional groups and interior pockets (Caminade et al., 2005; Tomalia, 2005). The non-polar pockets of dendrimers are responsive to encapsulation of guests with low aqueous solubility (Shi et al., 2010; Wang et al., 2011), while the surface functionalities can bind guests through covalent conjugates or non-covalent interactions such as ionic and hydrogen-bond interactions (D'Emanuele and Attwood, 2005). Large numbers of surface functionalities/interior pockets and large surface area endow dendrimers with high drug loading capacities (Cheng et al., 2011). Also, dendrimers are monomolecular micelles with excellent monodispersity and stability in physiological systems (Tomalia, 2005). As a result, they have been widely used as delivery vehicles for different families of drugs in the past decade (Agarwal et al., 2008, 2009; Gupta et al., 2006; Jain et al., 2010; Jain and Gupta, 2008). Isoflavones with phenolic groups and aromatic rings in their molecular structures, indicating these compounds can be either bound on the surface of cationic dendrimers via ionic interactions or encapsulated within the cavities through hydrophobic interactions.

In this study, we present an effective strategy to prepare liquid formulations of isoflavones, characterized the structures and physicochemical properties of the inclusion complexes of isoflavone and dendrimer, and evaluated their stability, cytotoxicity, and anti-oxidant activity. Isoflavones are present in multiple forms such as daidzein, genistein, glycitein, and their glycoside forms (Scheme 1) and the most investigated and beneficial isoflavones are daidzein and genistein. Here, daidzein was used as a model drug of isoflavones. Commercially available dendrimers poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) were used as potential carriers.

2. Experimental

2.1. Materials

Generation 3 (G3) and G5 ethylenediamine (EDA)-cored and amine-terminated PAMAM dendrimers were obtained from Dendritech Inc. (Midland, MI). G4 diaminobutane (DAB)-cored and amine-terminated PPI dendrimer was purchased from Sigma–Aldrich (St. Louis, MO). Daidzein was a gift from school of life science, East China Normal University. Dimethyl sulphoxide, acetic anhydride, and triethylamine were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). G3 and G5 PAMAM dendrimers were received in methanol solutions and the solvents were distilled to obtain the dendrimers in white gels. All the other chemicals were used as received without further purification.

2.2. Sample preparations

Fully acetylated G3 PAMAM (Ac-G3 PAMAM) and G4 PPI (Ac-G4 PPI) dendrimers were prepared according to a well-established method (Wang et al., 2011; Yang et al., 2008). Briefly, acetic anhydride (200% ratio of primary amine numbers of a G3 PAMAM or G4 PPI dendrimer with 32 surface amine groups) was slowly added to a dendrimer/methanol solution in the presence of triethylamine (1.25 molar equivalents of acetic anhydride). The mixtures were stirred for 24 h at room temperature. Acetic acid and triethylamine in the reaction solution were removed by extensive dialysis (MWCO is 3500 Da for Ac-G3 PAMAM and 1000 Da for Ac-G4 PPI) against PBS buffer and double-distilled water. Ac-G3 PAMAM and Ac-G4 PPI were obtained in white powders after lyophilization. The samples were stored in a dry place before further use. ^{1}H NMR spectroscopy was used to confirm the 100% acetylation ratio of the amine groups on G3 PAMAM and G4 PPI dendrimers.

Stock solutions for G3 and G5 PAMAM, Ac-G3 PAMAM and Ac-G4 PPI dendrimers were prepared at a concentration of 10 mg/mL in distilled water, while that for G4 PPI dendrimer were 20 mg/mL.

2.3. Phase solubility tests

Aqueous solubilities of daidzein in the absence and presence of PAMAM, PPI, and acetylated PAMAM and PPI dendrimers were measured. Generally, 2 mg daidzein was added into 400 μL distilled water or dendrimer solution in a tube. The mixtures were then shaken for 24 h at room temperature to ensure daidzein reach its saturated point in the solutions. Then the daidzein suspensions were centrifuged twice, the supernatants were kept and the daidzein concentrations in the supernatants were analyzed by a high performance liquid chromatography (HPLC) method. The dendrimer concentration in the solubility test ranges from 0 to 0.36 mM, and three repeats were conducted for each sample.

2.4. HPLC analysis

The concentration of daidzein was measured by an HPLC method as described elsewhere (Shen et al., 2010). Generally, the HPLC experiments were conducted on an HPLC instrument (Agilent1200, U.S.A.) equipped with a C18 column (4.6 mm diameter, 150 mm length, 5 μm particle size, ZORBAX Eclipse XDB, Agilent, U.S.A.). The mobile phase was methanol and water at a volume ratio of 50:50 and a flow rate of 1.0 mL/min. 10 μL daidzein solution was injected and the drug was detected at 254 nm. The retention time of daidzein is 6.9 ± 0.1 min. To prepare a standard curve for daidzein, the drug was dissolved in dimethyl sulphoxide at a concentration of 1 mg/mL and the solution was diluted to the concentration range of 0.5–75 μg/mL ($R \sim 0.9997$).

2.5. Stability test

The stability of daidzein in the absence and presence of G3 PAMAM and G4 PPI dendrimers was analyzed in a period of 30 d. Daidzein was dissolved in an 8 mL solution of dimethyl sulphoxide and distilled water (55:45, v/v) with or without 28.5 μM dendrimer. Daidzein concentrations in the samples were analyzed every 2 d by

an HPLC method. Three repeats were conducted for the stability of daidzein in the presence of PAMAM and PPI dendrimers.

2.6. Nuclear magnetic resonance (NMR) analysis

^{1}H NMR experiments on a Varian 699.804 MHz NMR spectrometer at 298.2 ± 0.1 K were conducted for G3 PAMAM/daidzein and G4 PPI/daidzein complexes in D_2O. Generally, 1 mg daidzein was dissolved in 500 μL dendrimer/D_2O solutions containing 1.14 mM PAMAM and PPI dendrimers. The solutions were sonicated for 2 h before NMR studies.

COSY spectrum of daidzein was obtained by the standard pulse program at Varian 699.804 MHz NMR spectrometer, with 1024 × 2048 data points. The relaxation delay was 1 s. 16 scans were averaged. A sine-bell squared window function and zero filling were applied to both dimensions. The TOCSY spectrum was obtained using an 8.4 μs, 90° pulse width, with a relaxation delay of 1 s. The mixing time was 80 ms. A spin-lock pulse was applied for the mixing period with a spin-lock field of 10 kHz with DIPSI-2 modulation. 8 scans were collected for each of the 512 increments in the indirect dimension. Both dimensions were processed using sine-bell window function and zero filling to a 1024 × 2048 matrix before Fourier transformation.

The ^{1}H–^{1}H two-dimensional nuclear Overhauser enhancement spectroscopy (2D-NOESY) experiments for G3 PAMAM/daidzein and G4 PPI/daidzein complexes were conducted on a Bruker Advance 500.132 MHz NMR instrument using standard pulse sequences. 1 s relaxation delay, 213 ms acquisition time, a 7.8 μs 90° pulse width, and a 300 ms mixing time were chosen. 16 transients were averaged for 800 complex t_1 points. All the data were processed with NMRpipe software on a Linux workstation.

2.7. In vitro release studies

The release rate of daidzein from G3 PAMAM or G4 PPI dendrimer was measured by the following procedures. Generally, PAMAM/daidzein and PPI/daidzein complexes were prepared by dissolving 150 μg daidzein in 1 mL PAMAM and PPI dendrimer solutions (0.29 mM), and the samples were sonicated for 2 h before the in vitro release studies. The dendrimer/daidzein complex solutions were immediately transferred into a dialysis bag with a molecular weight cut off (MWCO) of 1000 Da, followed by immersion of the dialysis bag into a container filled with 50 mL distilled water. 30 μL solutions from the outer phase of the dialysis bag were withdrawn at specific time intervals. The daidzein concentrations in these solutions were analyzed by an HPLC method.

2.8. Cell level studies

Cells were incubated in Dulbecoo's modified Eagle's medium (DMEM, GIBCO Inc.) containing streptomycin (100 μg/mL), penicillin sulphate (100 units/mL), and 10% heat-inactivated fetal calf serum (FCS, GIBCO Inc.). The cytotoxicities of G3 PAMAM and G4 PPI dendrimers and their complexes with daidzein on MCF-7 and A549 cells (ATCC) were measured by a well-established MTT assay. The cells were treated with dendrimers or dendrimer/daidzein complexes for 48 h (G3 PAMAM, 14.5 and 58.0 μM, G4 PPI 14.2 μM, and daidzein, 79 and 197 μM in the complexes), followed by the incubation of cells with MTT for 3 h. After that, the wells were added with dimethyl sulphoxide to dissolve the yielding purple crystals by living cells. The absorbance of the solution in each well was measured at 570 nm by a microplate reader (MQX200R, BioTeK Inc.).

The anti-oxidant activities of daidzein and dendrimer/daidzein complexes were conducted by evaluating the protective effect of these compounds on H_2O_2-induced cytotoxicity in MCF-7 and A549 cells (Heo et al., 2005). Generally, the cells were treated for 2 h with various concentrations of daidzein (75 μM and 187.5 μM dissolved by dimethyl sulphoxide) and its complexes with dendrimers (36.2 μM). The cells were then washed with PBS buffer and further treated with 1 μM H_2O_2 for 30 min. Viabilities of the cells treated with and without daidzein compounds were then measured by an MTT assay as described above. Six repeats were conducted for each sample and the results were shown as mean ± standard deviation. The data were analyzed by two-tailed, unpaired Student's *t*-tests.

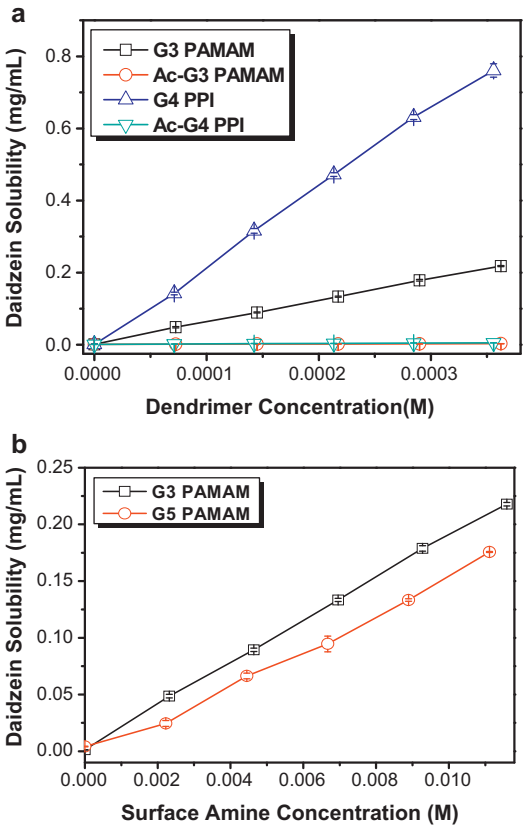


Fig. 1. Solubilities of daidzein in different concentrations of G3 PAMAM, G4 PPI, and acetylated G3 PAMAM and G4 PPI dendrimer solutions (a) and the comparison of daidzein solubility in G3 and G5 PAMAM dendrimer solutions (b).

3. Results and discussion

3.1. Daidzein loading by PAMAM and PPI dendrimers

As shown in Fig. 1a, the solubility of daidzein is extremely low in distilled water (1.17 μg/mL), and its solubility is significantly enhanced in the presence of both PAMAM and PPI dendrimers. The daidzein solubility is enhanced by 186 times and 650 times in 0.36 mM G3 PAMAM and G4 PPI dendrimer solutions, respectively, and the solubility increases linearly with dendrimer concentration. Previous study has reported that the solubility of daidzein was increased by 5.7-, 7.2-, and 9.4-fold by β-, methyl-β-, and hydroxypropyl-β-cyclodextrin, respectively (Borghetti et al., 2011). The use of binary systems containing cyclodextrin and hydrophilic polymers such as hydroxypropylmethylcellulose (HPMC) and polyvinylpyrrolidone (PVP) can further increase the solubility of daidzein (12.7-fold) (Borghetti et al., 2011). In separate studies, the solubility of daidzein was increased by 6–15 times depending on the cyclodextrin structure and concentration (Lei et al., 2005; Stancanelli et al., 2007). It is obvious that the increased solubility for daidzein still cannot meet the clinical need. Amphiphilic hyperbranched polyester with a dendritic structure

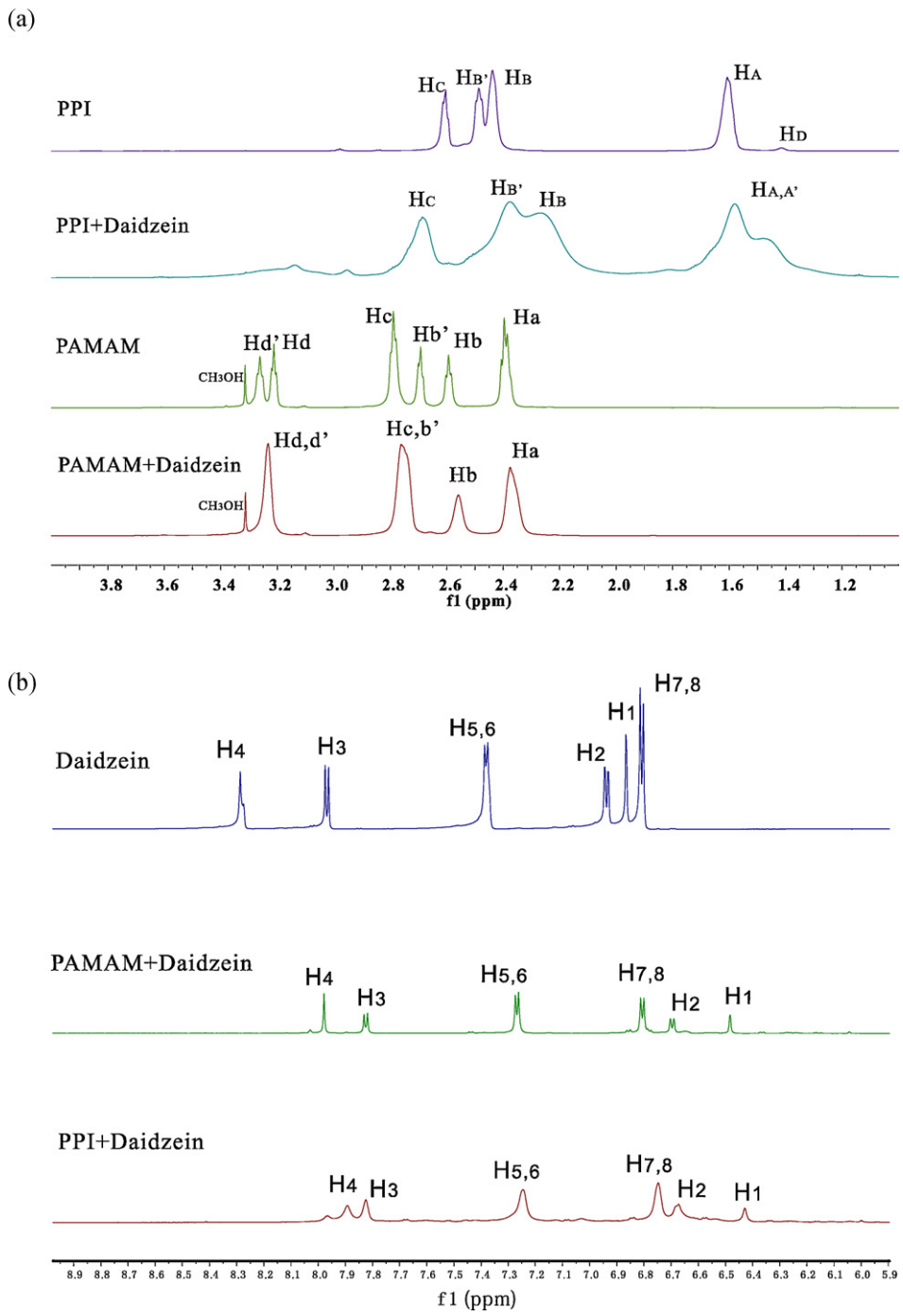


Fig. 2. ^{1}H NMR spectra of G3 PAMAM and G4 PPI dendrimers in the absence and presence of daidzein molecules, (a) the high field regions for dendrimers and (b) low field regions for daidzein.

improved the solubility of daidzein by 6–24 times at 5 mg/mL, indicating much lower loading efficiency of the dendritic polyester as compared to PAMAM (186-fold at 2.5 mg/mL) and PPI (650-fold at 1.25 mg/mL) dendrimers in the present study (Zou et al., 2005). The high solubilization behavior of PAMAM and PPI dendrimers towards daidzein presents an effective strategy to prepare liquid formulation of daidzein.

Both G3 PAMAM and G4 PPI dendrimers have 32 primary amine groups on their surface, the higher daidzein loading ability of G4 PPI than G3 PAMAM (Fig. 1a) should be attributed to be their differences in the interior structures of the two dendrimers (Kannaiyan and Imae, 2009). The interior pockets of PAMAM dendrimer are consisted of alkyl chain (–CH_2–CH_2–), tertiary amine, and amido groups (–CONH–), while the interior pockets of PPI dendrimer consisted of alkyl chain (–CH_2–CH_2–CH_2–) and tertiary amine group are much more hydrophobic than that of PAMAM dendrimer. The hydrophobic pockets of PPI dendrimer are involved in strong hydrophobic interactions with the aromatic region of the daidzein molecules (Richter-Egger et al., 2001). Therefore, G4 PPI encapsulates much more daidzein molecules than G3 PAMAM at the same molar concentration. The encapsulation of more daidzein molecules within PPI than that within PAMAM is further confirmed in later NMR investigations.

To investigate the importance of dendrimer surface amines on daidzein solubilization, we removed the surface amine groups of PAMAM and PPI dendrimers by acetylation (Wang et al., 2011; Yang

et al., 2008), and compared the solubilization behaviors of dendrimers before and after acetylation. Surprisingly, Ac-G3 PAMAM and Ac-G4 PPI dendrimers failed to load daidzein (Fig. 1a). As demonstrated in previous studies, the surface amine groups are proposed to be the predominant factor in the solubilization of hydrophobic drugs by cationic dendrimers (Cheng et al., 2008). Most of the surface amines of PAMAM and PPI should be protonated in the solubility studies ($pK_a \sim 10.5$; D'Emanuele and Attwood, 2005), pH ranges from 7.2 to 8.9 for PAMAM, and ($pK_a \sim 9.75$; Kannaiyan and Imae, 2009), and from 7.6 to 8.5 for PPI and strong ionic interactions between the phenolic groups of daidzein and the protonated amine occur on the surface of both dendrimers. The importance of surface ionic interaction on daidzein solubilization is further confirmed by the comparison of daidzein solubility in G3 and G5 PAMAM solutions. Our previous study has demonstrated that high generation dendrimer is more capable of encapsulation (more hydrophobic interior) and low generation dendrimer is much easier for surface ionic binding (lower steric hindrance) (Cheng et al., 2009). As shown in Fig. 1b, the daidzein solubilization ability of G3 PAMAM is higher than that of G5 PAMAM at the same surface amine concentration, indicating that surface ionic interaction plays an important role in the formation of PAMAM/daidzein complex. These results together suggest that the surface amine group is essential in the solubilization process and the ionic interaction is the first step of molecular encapsulation (Zhao et al., 2010).

3.2. NMR characterization of dendrimer/daidzein complex structures

To characterize the inclusion structure of PAMAM–daidzein and PPI–daidzein complexes, ^{1}H NMR, COSY, TOCSY, and 2D-NOESY techniques were employed. The proton chemical shift assignments of daidzein were carried out on the basis of ^{1}H NMR (Fig. 2), a homonuclear COSY and TOCSY spectra (Fig. 3), and the reference data (Whaley et al., 2006; Wu et al., 2009). COSY spectrum reveals *J*-coupled protons via cross-correlation peaks off the diagonal, while TOCSY spectrum creates correlations between all the protons within a given spin system, not limited to geminal or vicinal protons as in the COSY spectrum (Wu et al., 2009). In the molecular structure of daidzein (Scheme 1a), H_2, H_5, and H_6 are adjacent to H_3, H_7, and H_8, respectively. As shown in Fig. 3, cross-peaks are observed between $H_{5,6}/H_{7,8}$ and H_2/H_3 for daidzein in the absence and presence of PAMAM or PPI dendrimers, which allows a straightforward and unambiguous assignment of the chemical shifts for daidzein molecules in free states and in complex states with both dendrimers. An obvious change in the relationship between peaks $H_{1,2}$ and peak $H_{7,8}$ was observed in Fig. 3a and b, which is attributed to interactions between daidzein and dendrimers.

As shown in Fig. 2, significant downfield shifts of the dendrimer protons located on the outermost layer (protons $H_{b'}$ and $H_{d'}$ for PAMAM and protons H_C for PPI) were observed after the addition of daidzein molecules, indicating the ionic interactions between the amine groups of both dendrimers and the phenolic groups of daidzein (Hu et al., 2009). Also, obvious upfield shifts of protons H_A and H_B located in the interior of PPI dendrimer were observed (Fig. 2a), which is an evidence of PPI/daidzein inclusion formation via hydrophobic interactions. The broad peaks for daidzein molecules in the ^{1}H NMR spectrum of PPI/daidzein complex also confirmed the encapsulation of more daidzein molecules in PPI interior pockets (Fig. 2b). This result is in accordance with that obtained in phase-solubility studies.

^{1}H–^{1}H NOESY was further used to analyze the inclusion structure of the complexes (Figs. 4 and 5) (Zhao et al., 2010). Free daidzein with a molecular weight of 254 Da shows positive NOE signals, while G3 PAMAM and G4 PPI dendrimers (6900 and 3513 Da, respectively) exhibit negative NOE signals in a NOESY spectrum

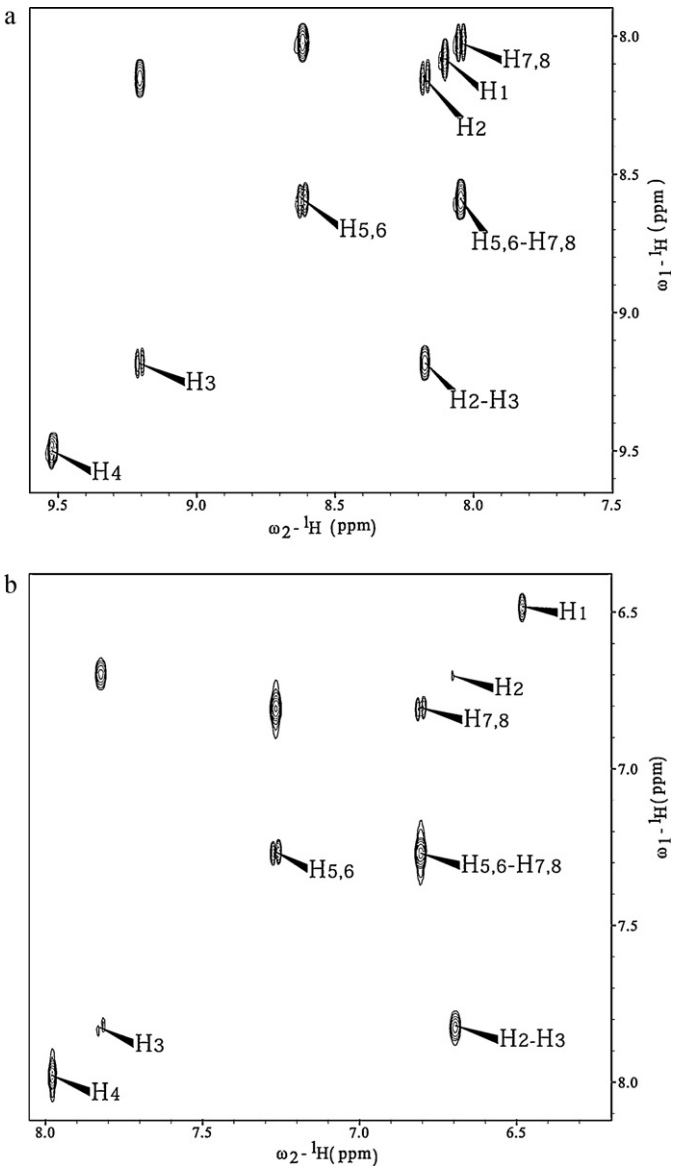
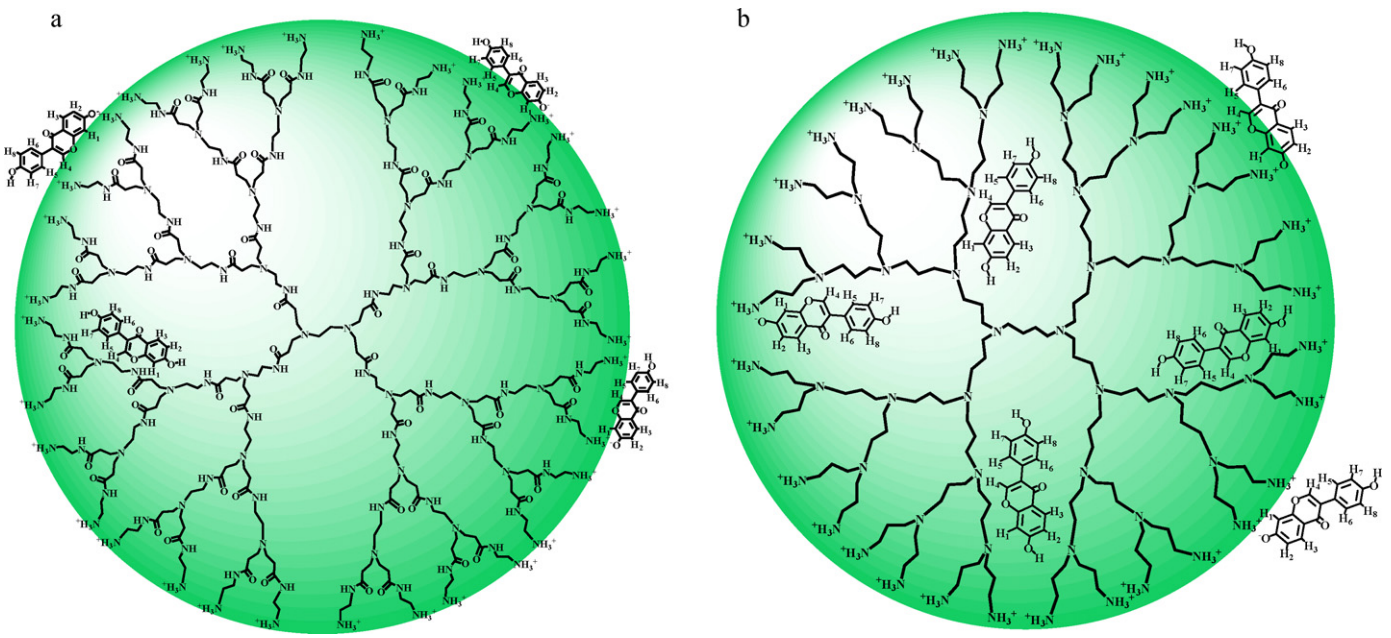


Fig. 3. Contour plots of the ^{1}H–^{1}H COSY spectra of daidzein in the absence (a) and presence (b) of G3 PAMAM dendrimer.

(Zhao et al., 2010). If daidzein molecules were bound with dendrimers, it develops a negative NOE signal for the bound-guests. The negative NOE signals (black) for daidzein in Figs. 4b and 5b indicate that most of the daidzein molecules were in bound-state. Weak cross-peaks between protons (H_4) of daidzein and protons ($H_{a,b,c}$) of G3 PAMAM dendrimer (Fig. 4a) and strong NOE cross-peaks between protons (H_{1-8}) of daidzein and protons (H_{A-C}) of G4 PPI dendrimer (Fig. 5a) were observed, proving the encapsulation of daidzein molecules within the cavities of both dendrimers. Obviously, the amount of daidzein molecules encapsulated within G4 PPI is much more than that within G3 PAMAM due to the more hydrophobic interior of PPI dendrimer. The weak cross-peaks between PAMAM and daidzein protons in Fig. 4a and the significant downfield shift of protons ($H_{b'}$ and $H_{d'}$) in Fig. 2a suggests that most of the daidzein molecules located on PAMAM surface via ionic interactions (Scheme 2). In the molecular structure of daidzein, protons H_2, and $H_{5,6}$ are adjacent to protons H_3 and $H_{7,8}$, respectively, therefore NOE cross-peaks for H_2–H_3, H_5–H_8, and H_6–H_7 are observed in Figs. 4b and 5b. However, cross-peaks for distant proton pairs (>5 Å) such as H_1–H_{7-8} and H_2–H_{7-8} are also observed in Fig. 5b,



Scheme 2. Proposed complex structures of daidzein with G3 PAMAM (a) and G4 PPI dendrimers (b).

indicating intermolecular NOE interactions for the daidzein molecules bound within the cavities of PPI dendrimers. In addition, the intensities of cross-peaks between protons $H_{1,4}$ of daidzein and protons of dendrimers (H_{a-d} of PAMAM and H_{A-D} of PPI) are much stronger than that between $H_{2,3}$ and dendrimer protons (Fig. 5a), providing insights into the average position and orientation of isoflavones within the dendrimer cavities. The approach of protons $H_{1,4}$ to the hydrophobic scaffold of dendrimers is attributed to fact that protons $H_{1,4}$ locate in the hydrophobic region of daidzein molecules (Scheme 2). The high binding ability of daidzein in the

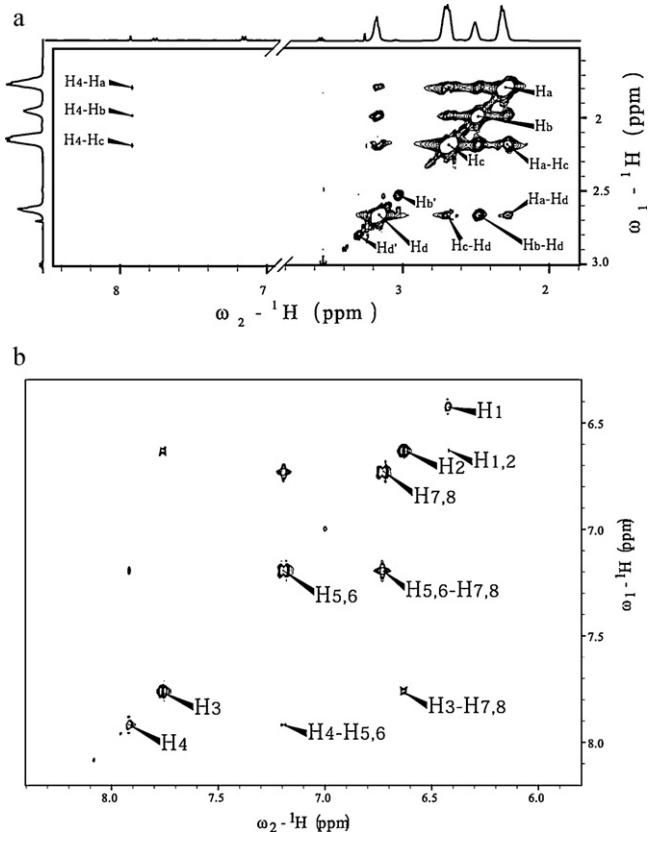


Fig. 4. Contour plots of the ^1H–^1H NOESY spectra of G3 PAMAM/daidzein complexes in D_2O at a mixing time of 300 ms. Expanded regions for G3 PAMAM/daidzein (a) and daidzein (b), respectively.

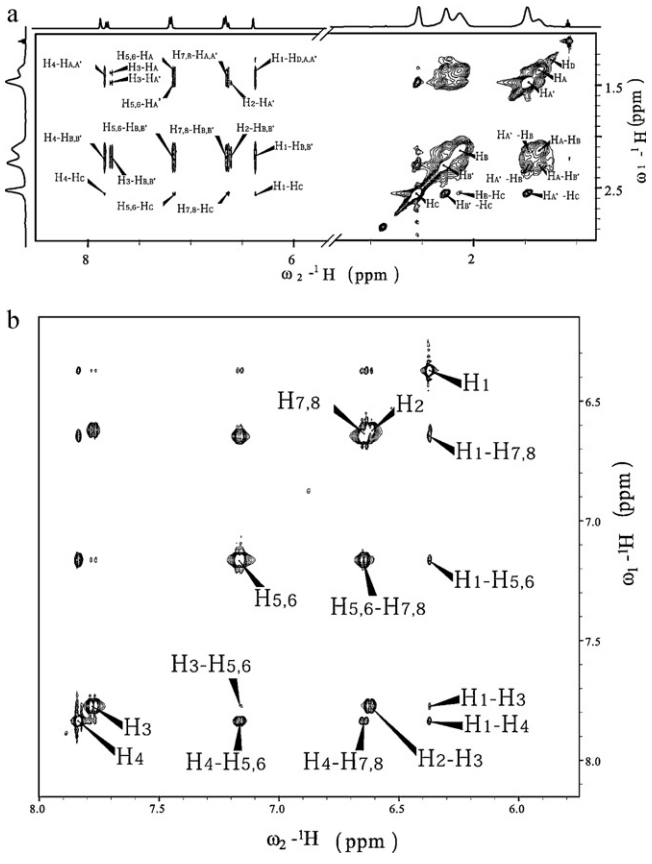


Fig. 5. Contour plots of the ^1H–^1H NOESY spectra of G4 PPI/daidzein complexes in D_2O at a mixing time of 300 ms. Expanded regions for G3 PAMAM/daidzein (a) and daidzein (b), respectively.

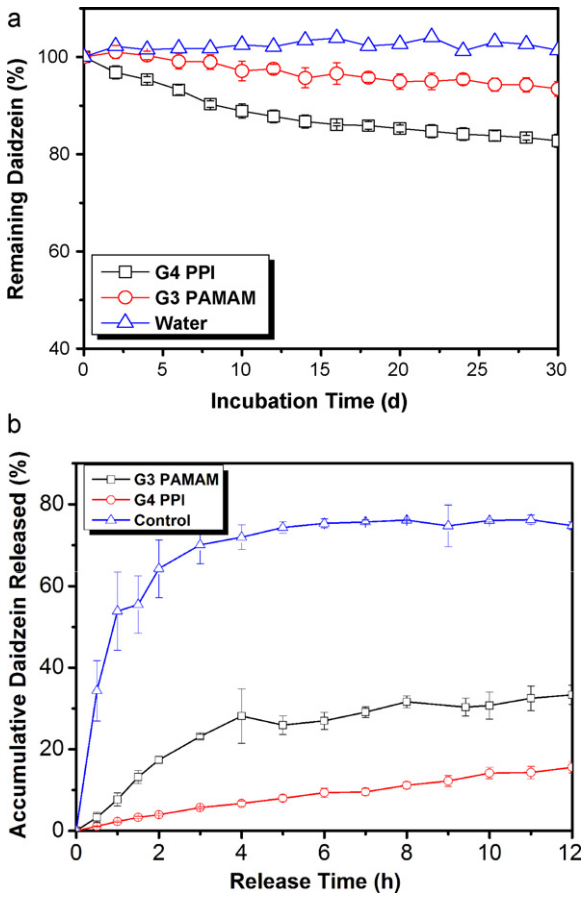


Fig. 6. The stability of daidzein in the absence and presence of G3 PAMAM and G4 PPI dendrimers during a period of 30 d (a) and the release behaviors of daidzein molecules from G3 PAMAM/daidzein and G4 PPI/daidzein complexes (b).

cavities of G4 PPI dendrimer well explains the high solubility of daidzein in PPI solutions.

3.3. Stability of daidzein in the presence of PAMAM and PPI dendrimers

Drug stability is an important issue of a formulation, which should be considered before clinical applications. Formulations with physical and chemical stability can maintain the long-term therapeutic efficacy of the drug. To investigate the stability of daidzein in the inclusion complexes with G3 PAMAM and G4 PPI dendrimer, the degradation of daidzein molecules in the absence and presence of both dendrimers were monitored by HPLC for a month. As shown in Fig. 6a, 94% and 84% of added daidzein molecules were recovered after 30 d in the presence of G3 PAMAM and G4 PPI dendrimers, respectively. Daidzein and other isoflavones were reported to cause supramolecular aggregates of phospholipid vesicles (Whaley et al., 2006). The decreased daidzein in the dendrimer/daidzein complexes is probably due to the formation of supramolecular aggregates, inclusions, or precipitates between cationic dendrimers and daidzein with two negatively charged groups (Hu et al., 2009; Zhao et al., 2009). G4 PPI dendrimer has a more hydrophobic interior and a smaller size (2.3 nm versus 3.0 nm) than G3 PAMAM, which makes the supramolecular aggregate or inclusion of G4 PPI/daidzein not as soluble as that of G3 PAMAM/daidzein. The comparison of the two dendrimer/daidzein complexes in Fig. 6 clearly demonstrates that G3 PAMAM/daidzein has better stability and can be stored for a longer period than G4 PPI/daidzein.

3.4. In vitro release behaviors of daidzein from PAMAM and PPI dendrimers

The in vitro release experiments were carried out to evaluate the sustained release characteristic of the dendrimer/daidzein complexes, which is of critical importance in the design and optimization of drug delivery systems. Carriers with ideal sustained release characteristics can improve bioavailability and decrease side-effects of the administrated drug, and simplify the dosing schedules (Cheng et al., 2011). As shown in Fig. 6b, the accumulative percent of drugs released from PAMAM/daidzein complex is 27.0% at 6 h and 33.4% at 12 h, while the corresponding values are 9.3% and 15.6% for PPI/daidzein complex. The release rate of daidzein from the dendrimer/daidzein complexes is much slower than that from an amphiphilic hyperbranched polyester system, in which more than 50% daidzein was released out of the dendritic polymer during a period of 10 h (Zou et al., 2005). In the case of daidzein/phospholipid complex (Zhang et al., 2011) and daidzein/solid lipid nanoparticles (Gao et al., 2008), the accumulative release of daidzein from the nanoparticles was about 60% within 8 h (Zhang et al., 2011) and 45% within 12 h (Gao et al., 2008), respectively. Therefore, both PAMAM and PPI dendrimers are better candidates in the design of sustained release systems for isoflavones.

As demonstrated in phase-solubility and NMR studies, G4 PPI has higher solubilization ability towards daidzein than G3 PAMAM, indicating that G4 PPI has more binding sites for daidzein molecules. More binding sites in a carrier can prevent the rapid release of drugs from the dendrimer/drug complexes (Hu et al., 2009). In addition, NOESY studies have proved that more daidzein were encapsulated within the cavities of G4 PPI than that within G3 PAMAM. Strong hydrophobic interactions between the PPI scaffold and the aromatic rings of daidzein further slow the release rate of daidzein. Therefore, G4 PPI shows better sustained release behavior than G3 PAMAM (Fig. 6b).

3.5. Cytotoxicities of dendrimer/daidzein complexes

The cytotoxicities of G3 PAMAM/daidzein and G4 PPI/daidzein complexes were evaluated on MCF-7 and A549 cells by an MTT assay. As shown in Fig. 7a and b, daidzein at 79–197 μM and G3 PAMAM dendrimer at 14.5 μM are not toxic on both cells. However, 14.2 μM G4 PPI causes a 63% and 81.2% cell death on MCF and A549 cells, respectively, suggesting high cytotoxicity of G4 PPI dendrimer on both cells. Dendrimer surface charge was proposed to be the predominant factor on the cytotoxicity of dendrimer (Duncan and Izzo, 2005; Jain et al., 2010; Mishra et al., 2009, 2010). However, both G3 PAMAM and G4 PPI have 32 primary amine groups on dendrimer surface. Therefore, the higher cytotoxicity of G4 PPI is probably attributed to its more hydrophobic interior as compared to G3 PAMAM. The hydrophobic interior of G4 PPI is able to encapsulate the hydrophobic region of phospholipid, which disturbs the bilayer structure of the cell membrane (Smith et al., 2010). Also, dendrimer with a more hydrophobic interior has higher penetration ability through the cell membrane, thus exhibiting a higher cytotoxicity on the cells. Though the loading capacity of G4 PPI is higher than that of G3 PAMAM (~3.5 fold), the cytotoxicity of G4 PPI is much higher than that of G3 PAMAM, since G3 PAMAM at a high concentration of 72.5 μM is non-toxic on MCF-7 and A549 cells, while G4 PPI exhibits serious cytotoxicity on both cells at 14.2 μM. Besides, the G3 PAMAM/daidzein complex (14.5 μM/79 μM) is not toxic on both cells, while G4 PPI/daidzein complex at a similar concentration killed most of cells, suggesting that G3 PAMAM/daidzein complex is a safer choice than G4 PPI/daidzein complex in the delivery of daidzein.

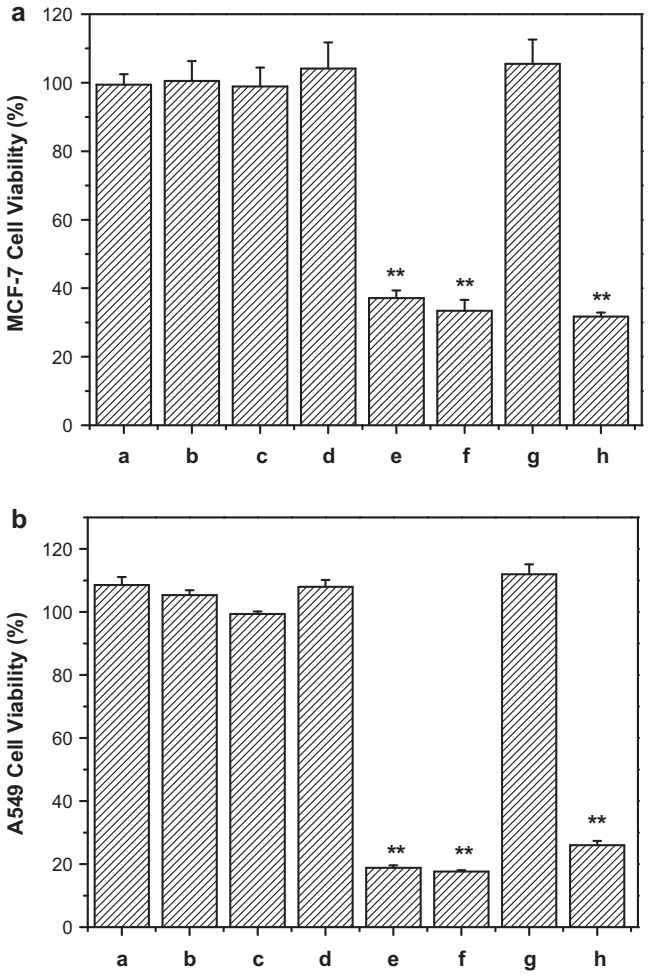


Fig. 7. Viability of MCF-7 (a) and A549 cells (b) incubated with G3 PAMAM, G4 PPI, daidzein, and G3 PAMAM/daidzein and G4 PPI/daidzein complexes for 48 h, a: 14.5 μM PAMAM, b: 79 μM daidzein, c: 14.5 μM PAMAM + 79 μM daidzein, d: 72.5 μM PAMAM, e: 14.2 μM PPI, f: 14.2 μM PPI + 79 μM daidzein, g: 197 μM daidzein, and h: 14.2 μM PPI + 197 μM daidzein, $^{**}p < 0.05$, compared to the cell viability of G3 PAMAM at 14.5 μM.

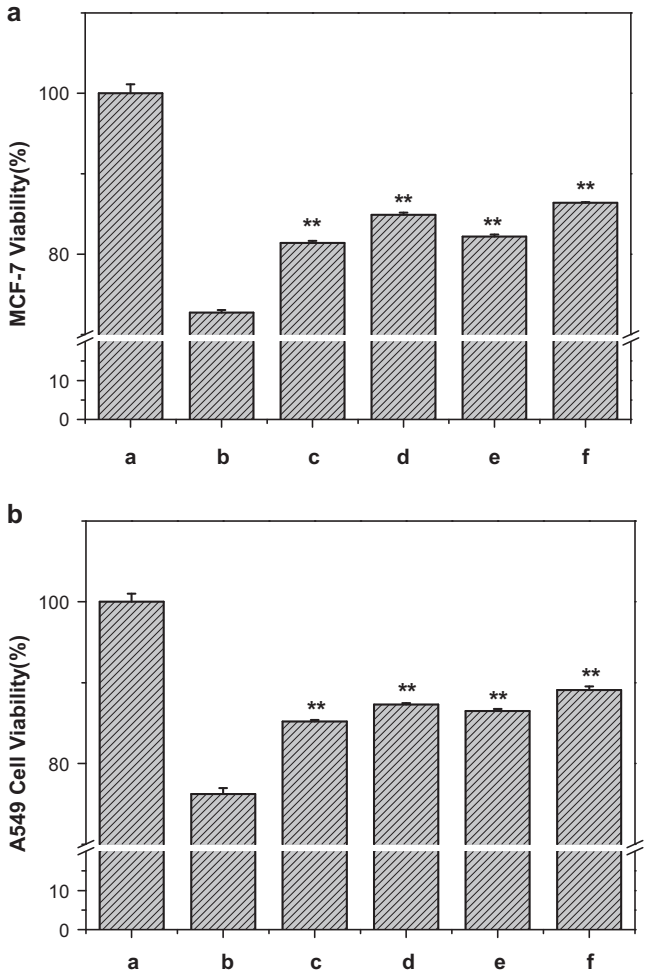


Fig. 8. Protective effect of daidzein and daidzein/G3 PAMAM complexes on H_2O_2-induced cytotoxicity in (a) MCF-7 and (b) A549 cells, a: untreated, b: 1 μM H_2O_2, c: 1 μM H_2O_2 + 75 μM daidzein/36.2 μM PAMAM complex, d: 1 μM H_2O_2 + 187.5 μM daidzein/36.2 μM PAMAM complex, e: 1 μM H_2O_2 + 75 μM daidzein, and f: 1 μM H_2O_2 + 187.5 μM daidzein, $^{**}p < 0.05$, compared to the viability of 1 μM H_2O_2 treated cells.

3.6. Anti-oxidant activities of daidzein and dendrimer/daidzein complexes

Daidzein and other isoflavones were reported to have anti-oxidant properties by enhancing the activities of catalase, superoxide dismutase and glutathione reductase (Barbosa et al., 2011; Kim et al., 2004; Valsecchi et al., 2011). To investigate the anti-oxidant activity of daidzein in the formulations of dendrimer/daidzein complexes, protective effect of these compounds on H_2O_2-induced cytotoxicity in MCF-7 and A549 cells were evaluated (Heo et al., 2005). Since G4 PPI/daidzein complexes showed significant cytotoxicity on both MCF-7 and A549 cells, only the anti-oxidant activities of G3 PAMAM/daidzein complexes were evaluated. Incubation of both cells with 1 μM H_2O_2 for 30 min causes a significant decrease in cell viability (72.7 ± 0.3% on MCF-7 cells and 76.2 ± 0.7% on A549 cells, Fig. 8a and b), but the presence of daidzein (187.5 μM) inhibited the H_2O_2-induced cytotoxicity on both cells (86.4 ± 0.1% on MCF-7 cells and 89.1 ± 0.5% on A549 cells). G3 PAMAM/daidzein complexes showed similar protective effects as free daidzein at the same molar concentrations of the compound (84.9 ± 0.3% on MCF-7 cells and 87.3 ± 0.2% on A549 cells, Fig. 8a and b), suggesting that the complexation of daidzein to G3 PAMAM dendrimer does not significantly influence the bioactivity of the compound. Pretreatment of the cells with increasing concentrations of daidzein or dendrimer/daidzein complexes showed improved protective effect. These results suggest that dendrimer/daidzein complexes have potential applications as anti-oxidant agents for therapeutic purpose and the bioactivities of these formulations can be tailored by varying daidzein concentrations.

4. Conclusions

In the present study, the inclusion complexes of daidzein and two commercially available dendrimers were prepared and characterized. Both PAMAM and PPI dendrimers remarkably improved the aqueous solubility of daidzein. PPI shows a better drug loading capacity than PAMAM due to its more hydrophobic interior. The interaction mechanisms between both dendrimer and daidzein were proposed based on the phase-solubility studies. NMR analysis proved the inclusion structure of both dendrimer/daidzein complexes and the encapsulation of more daidzein molecules within the cavities of PPI dendrimer. Daidzein exhibits a slower release rate from the PPI/daidzein complex than that from the PAMAM/daidzein complex. However, PPI is much more toxic than PAMAM on MCF-7 and A549 cells. The stability of PAMAM/daidzein complex is better

than that of PPI/daidzein complex. G3 PAMAM/daidzein complex showed similar protective effect on H_2O_2-induced cytotoxicity on MCF-7 and A549 cells as free daidzein. Therefore, the inclusion of daidzein with dendrimer can effectively improve the solubility, prolong the delivery, and maintain the bioactivity of daidzein. Though PAMAM showed a lower daidzein loading capacity compared with PPI, it is a safer choice than PPI in the design of polymeric delivery systems for daidzein. This research provides new insights into dendrimer-based drug delivery systems and will be helpful for the design of novel dendrimer/drug formulations.

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References

Agarwal, A., Asthana, A., Gupta, U., Jain, N.K., 2008. Tumour and dendrimers: a review on drug delivery aspects. J. Pharm. Pharmacol. 60, 671–688.

Agarwal, A., Gupta, U., Asthana, A., Jain, N.K., 2009. Dextran conjugated dendritic nanoconstructs as potential vectors for anti-cancer agent. Biomaterials 30, 3588–3596.

Barbosa, A.C., Lajolo, F.M., Genovese, M.I., 2011. Effect of free or protein-associated soy isoflavones on the antioxidant status in rats. J. Sci. Food Agric. 91, 721–731.

Borghetti, G.S., Pinto, A.P., Lula, I.S., Sinisterra, R.D., Teixeira, H.F., Bassani, V.L., 2011. Daidzein/cyclodextrin/hydrophilic polymer ternary systems. Drug Dev. Ind. Pharm. 37, 886–893.

Byun, J.S., Lee, S.S., 2010. Effect of soybeans and sword beans on bone metabolism in a rat model of osteoporosis. Ann. Nutr. Metab. 56, 106–112.

Caminade, A.M., Laurent, R., Majoral, J.P., 2005. Characterization of dendrimers. Adv. Drug Deliv. Rev. 57, 2130–2146.

Cannava, C., Crupi, V., Ficarra, P., Guardo, M., Majolino, D., Mazzaglia, A., Stancanelli, R., Venuti, V., 2010. Physico-chemical characterization of an amphiphilic cyclodextrin/genistein complex. J. Pharm. Biomed. Anal. 51, 1064–1068.

Cheng, Y.Y., Li, Y.W., Wu, Q.L., Zhang, J.H., Xu, T.W., 2009. Generation-dependent encapsulation/electrostatic attachment of phenobarbital molecules by poly(amidoamine) dendrimers: evidence from 2D-NOESY investigations. Eur. J. Med. Chem. 44, 2219–2223.

Cheng, Y.Y., Wu, Q.L., Li, Y.W., Xu, T.W., 2008. External electrostatic interaction versus internal encapsulation between cationic dendrimers and negatively charged drugs: which contributes more to solubility enhancement of the drugs? J. Phys. Chem. B 112, 8884–8890.

Cheng, Y.Y., Zhao, L.B., Li, Y.W., Xu, T.W., 2011. Design of biocompatible dendrimers for cancer diagnosis and therapy: current status and future perspectives. Chem. Soc. Rev. 40, 2673–2703.

D'Emanuele, A., Attwood, D., 2005. Dendrimer–drug interactions. Adv. Drug Deliv. Rev. 57, 2147–2162.

Duncan, R., Izzo, L., 2005. Dendrimer biocompatibility and toxicity. Adv. Drug Deliv. Rev. 57, 2215–2237.

Gao, Y., Gu, W., Chen, L., Xu, Z., Li, Y.P., 2008. The role of daidzein-loaded sterically stabilized solid lipid nanoparticles in therapy for cardio-cerebrovascular diseases. Biomaterials 29, 4129–4136.

Ge, Y.B., Chen, D.W., Xie, L.P., Zhang, R.Q., 2007. Optimized preparation of daidzein-loaded chitosan microspheres and in vivo evaluation after intramuscular injection in rats. Int. J. Pharm. 338, 142–151.

Gupta, U., Agashe, H.B., Asthana, A., Jain, N.K., 2006. Dendrimers: novel polymeric nanoarchitectures for solubility enhancement. Biomacromolecules 7, 649–658.

Heo, H.J., Kim, M.J., Suh, Y.M., Choi, S.J., Mun, N.S., Kim, H.K., Kim, E.K., Shin, D.H., 2005. Protective effects of daidzein on oxidative stress-induced neurotoxicity and scopolamine-mediated cognitive defect. J. Food Sci. 70, S91–S94.

Hu, J.J., Cheng, Y.Y., Ma, Y.R., Wu, Q.L., Xu, T.W., 2009. Host–guest chemistry and physico-chemical properties of dendrimer–mycophenolic acid complexes. J. Phys. Chem. B 113, 64–74.

Jain, K., Kesharwani, P., Gupta, U., Jain, N.K., 2010. Dendrimer toxicity: let's meet the challenge. Int. J. Pharm. 394, 122–142.

Jain, N.K., Gupta, U., 2008. Application of dendrimer–drug complexation in the enhancement of drug solubility and bioavailability. Expert Opin. Drug Metab. Toxicol. 4, 1035–1052.

Jiang, Q., Payton-Stewart, F., Elliott, S., Driver, J., Rhodes, L.V., Zhang, Q., Zheng, S., Bhatnagar, D., Boue, S.M., Collins-Burow, B.M., Sridhar, J., Stevens, C., McLachlan, J.A., Wiese, T.E., Burow, M.E., Wang, G., 2010. Effects of 7-O substitutions on estrogenic and anti-estrogenic activities of daidzein analogues in MCF-7 breast cancer cells. J. Med. Chem. 53, 6153–6163.

Kannaiyan, D., Imae, T., 2009. pH-dependent encapsulation of pyrene in PPI-core: PAMAM-shell dendrimers. Langmuir 25, 5282–5285.

Kim, S.Y., Kim, S.J., Lee, J.Y., Kim, W.G., Park, W.S., Sim, Y.C., Lee, S.J., 2004. Protective effects of dietary soy isoflavones against UV-induced skin-aging in hairless mouse model. J. Am. Coll. Nutr. 23, 157–162.

Lee, S.H., Kim, Y.H., Yu, H.J., Cho, N.S., Kim, T.H., Kim, D.C., Chung, C.B., Hwang, Y.I., Kim, K.H., 2007. Enhanced bioavailability of soy isoflavones by complexation with beta-cyclodextrin in rats. Biosci. Biotechnol. Biochem. 71, 2927–2933.

Lei, Y.J., Yu, M., Zhao, K., 2005. Preparation and characterization of inclusion complex of daidzein with beta-cyclodextrin. Food Sci. 26, 134–137.

Mishra, V., Gupta, U., Jain, N.K., 2009. Surface-engineered dendrimers: a solution for toxicity issues. J. Biomater. Sci. Polym. Ed. 20, 141–166.

Mishra, V., Gupta, U., Jain, N.K., 2010. Influence of different generations of poly(propylene imine) dendrimers on human erythrocytes. Pharmazie 65, 891–895.

Reiter, E., Reiter, E., Beck, V., Medjakovic, S., Jungbauer, A., 2009. Isoflavones are safe compounds for therapeutical applications—evaluation of in vitro data. Gynecol. Endocrinol. 25, 554–580.

Richter-Egger, D.L., Tesfai, A., Tucker, S.A., 2001. Spectroscopic investigations of poly(propyleneimine) dendrimers using the solvatochromic probe phenol blue and comparisons to poly(amidoamine) dendrimers. Anal. Chem. 73, 5743–5751.

Shen, Q., Li, X., Yuan, D., Jia, W., 2010. Enhanced oral bioavailability of daidzein by self-microemulsifying drug delivery system. Chem. Pharm. Bull. 58, 639–643.

Shi, X.Y., Lee, I.H., Chen, X.S., Shen, M.W., Xiao, S.L., Zhu, M.F., Baker Jr., J.R., Wang, S.H., 2010. Influence of dendrimer surface charge on the bioactivity of 2-methoxyetradiol complexed with dendrimers. Soft Matter 6, 2539–2545.

Smith, P.E.S., Brender, J.R., Ulrich, H., Durr, N., Xu, J.D., Mullen, D.G., Banaszak, Holl, M.M., Ramamoorthy, A., 2010. Solid-state NMR reveals the hydrophobic-core location of poly(amidoamine) dendrimers in biomembranes. J. Am. Chem. Soc. 132, 8087–8097.

Sojitra, P., Raval, A., Kothwala, D., Kotadia, H., Adeshara, 2010. Covalently conjugation of genistein with biodegradable poly L-lactide. Trends Biomater. Artif. Organs 23, 144–148.

Stancanelli, R., Mazzaglia, A., Tomamasini, S., Calabro, M.L., Villari, V., 2007. The enhancement of isoflavones water solubility by complexation with modified cyclodextrins: a spectroscopic investigation with implications in the pharmaceutical analysis. J. Pharm. Biomed. Anal. 44, 980–984.

Tomalia, D.A., 2005. Birth of a new macromolecular architecture: dendrimers as quantized building blocks for nanoscale synthetic polymer chemistry. Prog. Polym. Sci. 30, 294–324.

Valsecchi, A.E., Franchi, S., Panerai, A.E., Rossi, A., Sacerdote, P., Colleoni, M., 2011. The soy isoflavone genistein reverses oxidative and inflammatory state, neuropathic pain, neurotrophic and vasculature deficits in diabetes mouse model. Eur. J. Pharmacol. 650, 694–702.

Wang, Y., Guo, R., Cao, X., Shen, M., Shi, X.Y., 2011. Encapsulation of 2-methoxyestradiol within multifunctional poly(amidoamine) dendrimers for targeted cancer therapy. Biomaterials 32, 3322–3329.

Ward, H.A., Kuhnle, G.G., 2010. Phytoestrogen consumption and association with breast, prostate and colorectal cancer in EPIC Norfolk. Arch. Biochem. Biophys. 501, 170–175.

Warri, A., Saarinen, N.M., Makela, S., Hilakivi-Clarke, L., 2008. The role of early life genistein exposures in modifying breast cancer risk. Br. J. Cancer 98, 1485–1493.

Whaley, W.L., Rummel, J.D., Kastrapeli, N., 2006. Interaction of genistein and related isoflavones with lipid micelles. Langmuir 22, 7175–7184.

Wu, Q.L., Cheng, Y.Y., Hu, J.J., Zhao, L.B., Xu, T.W., 2009. Insights into the interactions between dendrimers and bioactive surfactants: 3. Size-dependent and hydrophobic property-dependent encapsulation of bile salts. J. Phys. Chem. B 113, 12934–12943.

Xavier, C.R., Silva, A.P.C., Schwingel, L.C., Borghetti, G.S., Koester, B.L., Mayorga, P., Teixeira, H.F., Bassani, V.L., 2010. Improvement of genistein content in solid genistein/beta-cylcodextrin complexes. Quim. Nova 33, 587–590.

Yang, W.J., Cheng, Y.Y., Xu, T.W., Wang, X.Y., Wen, L.P., 2008. Targeting cancer cells with biotin–dendrimer conjugates. Eur. J. Med. Chem. 44, 862–868.

Zhang, Z.W., Huang, Y., Gao, F., Bu, H.H., Gu, W.W., Li, Y.P., 2011. Daidzein–phospholipid complex loaded lipid nanocarriers improved oral absorption: in vitro characteristics and in vivo behavior in rats. Nanoscale 3, 1780–1787.

Zhao, L.B., Cheng, Y.Y., Hu, J.J., Wu, Q.L., Xu, T.W., 2009. Host–guest chemistry of dendrimer–drug complexes. 3. Competitive binding of multiple drugs by a single dendrimer for combination therapy. J. Phys. Chem. B 113, 14172–14179.

Zhao, L.B., Wu, Q.L., Cheng, Y.Y., Zhang, J.H., Wu, J.H., Xu, T.W., 2010. High-throughput screening of dendrimer-binding drugs. J. Am. Chem. Soc. 132, 13182–13184.

Zou, J.H., Shi, W.F., Wang, J., Bo, J., 2005. Encapsulation and controlled release of a hydrophobic drug using a novel nanoparticle-forming hyperbranched polyester. Macromol. Biosci. 5, 662–668.