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Full Paper

Host–Guest Inclusion System of Luteolin with Polyamine-β-cyclodextrin: Preparation, Characterisation, Anti-oxidant and Anti-cancer Activity

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The characterisation, inclusion complexation behaviours, and binding ability of inclusion complexes of luteolin (LU) with four polyamine-modified β-cyclodextrins (NH_2-βCD, EN-βCD, DETA-βCD, TETA-βCD; where EN = ethylenediamine; DETA = diethylenediamine; TETA = triethylenetetramine) were investigated in both the solid and solution forms by photoluminescence spectroscopy, ^{1}H and 2D NMR spectroscopy, differential scanning calorimetry, X-ray diffraction, and scanning electron microscopy. The results showed that the water solubility, and the anti-oxidant activity and anti-cancer activity of LU were significantly increased in the inclusion complex with polyamine-β-cyclodextrin. The LU/CDs complex will be useful for its application as herbal medicine or healthcare product.

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Introduction

Luteolin (LU; Chart 1), a 3′,4′,5,7-tetrahydroxyflavone, is widely found in a variety of plants, especially vegetables such as celery, green pepper, and Perilla leaves.[1] LU exhibits a wide range of pharmacological activities, including anti-inflammation, anti-microbial, anti-cancer, anti-allergic, cardio-protective properties,[2–6] and other metabolic disorders.[7] It is well known that LU possesses strong anti-oxidative activity, even stronger than vitamin E and butylated hydroxytoluene.[8] Consequently, numerous studies reported the inhibition effect against a wide range of oxidative stress-associated pathologies such as cancer and heart diseases. However, its poor solubility (1.93×10^{-5} mol kg^{-1} at 20°C) and stability in water severely restrict its application as a drug. Therefore, it is necessary to find an efficient and non-toxic carrier for LU to further its clinical application.

It is known that cyclodextrins (CDs) are truncated-cone polysaccharides that are mainly made up of six-to-eight D-glucose monomers linked by α-1,4-glycosidic bonds. Additionally, they have a hydrophilic exterior and a hydrophobic cavity.[9] They form inclusion complexes with various hydrophobic molecules.[10,11] Recently, Ficarra et al. and Duncan et al. reported the inclusion complex formation of CDs and flavonoids.[12,13] However, the unmodified or substituted β-CD has relatively poor water solubility and may be at risk of nephrotoxicity.[14] To tackle these problems, modified β-CD was applied.[15]

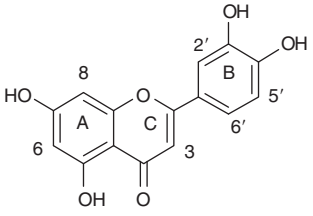

Chart 1. The structure of luteolin.

The chemically modified β-CDs can largely alter the original molecular-binding ability by stereochemical complementarity of the functional branch located in the CD cavity.[16–19] Encouragingly, the complexation of LU with β-CD, HP-βCD, DM-βCD, and SBE-βCD has been achieved (HP-βCD = hydroxypropy-β-cyclodextrin; DM-βCD = carboxymethyl-β-cyclodextrin; SBE-βCD = sulfobutyl ether-β-cycoldextrin).[20] Meanwhile, other types of CDs as molecular hosts for LU have not been well explored. LU is a weak acid flavonoid and polyamine-modified CDs are alkaline. Thus, we anticipate that the binding affinity between polyamine-modified CDs and LU will be slightly stronger than that between native β-CD and LU.

We herein reported the preparation and characterisation of the inclusion complexes of LU with polyamine-modified CDs with different tethered chain lengths i.e. amino-β-cyclodextrin (NH_2-βCD), ethylenediamine-β-cyclodextrin (EN-βCD), diethylenetriamine-β-cyclodextrin (DETA-βCD), and triethylenetetramine-β-cyclodextrin (TETA-βCD) (Chart 2). The binding behaviour of the water-soluble polyamine-modified CDs with LU, and the anti-oxidant and anti-cancer activity of LU after formation of the inclusion complexes were also investigated. We believe that such LU polyamine-modified CDs complexes could potentially be applied in healthcare

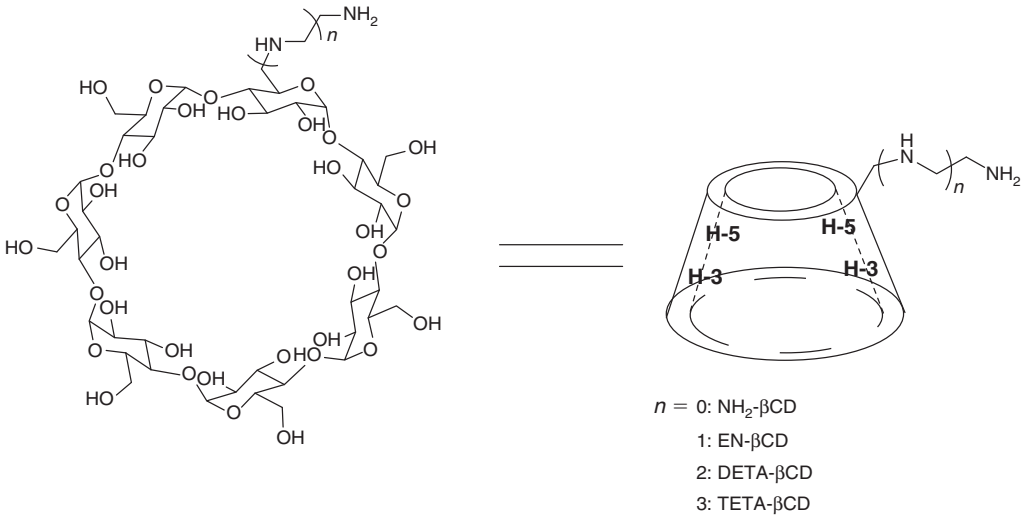

Chart 2. The structure of the polyamine-β-cyclodextrins.

products upon further development. To our knowledge, this is the first investigation of the inclusion behaviour of LU and polyamine-modified CDs complexes.

Result and Discussion

Stoichiometry

The stoichiometry for the inclusion complex of LU with CDs was determined by the Job's method. Fig. 1 illustrates the Job plot for the LU/EN-βCD system. The plot for EN-βCD showed a maximum value at a molar fraction of 0.5, which indicated the 1 : 1 inclusion complexation between LU and CDs. Similar results were obtained for the complexes of LU with β-CD, NH_2-βCD, DETA-βCD, and TETA-βCD. The results were consistent with the previous study by Liu who used the phase solubility method.[21]

Spectral Titration

The inclusion complex binding behaviour between host β-CDs and LU was studied in phosphate buffered solution using fluorescence spectroscopy. The complex stability constants (K_S) were determined by changes in the absorbance intensity upon addition of the host molecule. As the Job plot showed the stoichiometry for the inclusion complexes of β-CDs with the guest molecule LU is 1 : 1, the inclusion complex of host (H) with guest (G) was expressed by Eqn 1.

$$H + G \overset{K_S}{\rightleftharpoons} H \cdot G \tag{1}$$

Parameter K_S was calculated according to Eqn 2.

$$K_S = \frac{[LU \bullet CD]}{[LU][CD]} = \frac{\Delta F/\Delta\varepsilon}{\left([CD]_0 - \Delta F/\Delta\varepsilon\right)\left([LU]_0 - \Delta F/\Delta\varepsilon\right)} \tag{2}$$

Here $[CD]_0$ and $[LU]_0$ refer to the total concentration of CD and LU, and ΔF can be expressed by Eqn 3.

$$\Delta F = \frac{(\Delta\varepsilon)([CD]_0 + [LU]_0 + 1/K_S) \pm \sqrt{(\Delta\varepsilon)^2([CD]_0 + [LU]_0 + 1/K_S)^2 - 4(\Delta\varepsilon)^2[CD]_0[LU]_0}}{2} \tag{3}$$

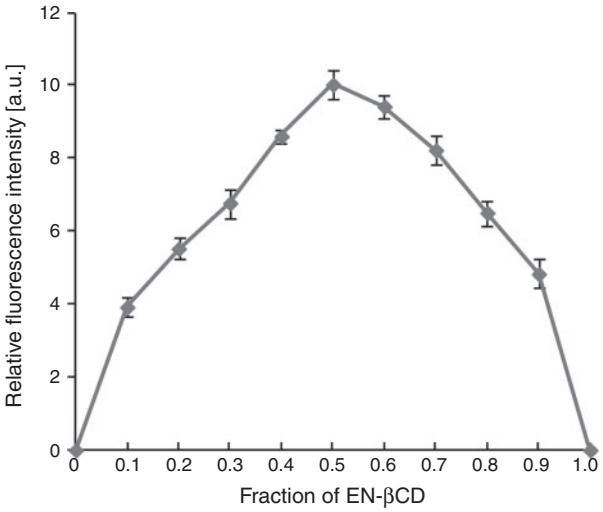

Fig. 1. Job plot of the LU/EN-βCD system at λ_{em} 430 nm ([LU]+[EN-βCD] = 4.0×10^{-5} M) obtained in pH 7.4 buffer.

Parameter $\Delta\varepsilon$ is the proportionality coefficient, which may be regarded as a sensitivity factor for the fluorescence intensity change.

As illustrated in Fig. 2, the fluorescence intensity of LU increased with the gradual addition of CDs. The fluorescence quantum yield could be improved by formation of an inclusion complex.[22] By using a non-linear least-squares curve-fitting method, the complex constant could obtained.[23] It also showed the perfect fit between the calculated and experimental data. For all the hosts examined, a perfect fit was obtained for the plot of ΔF as a function of $[G]_0$ (total concentration of guest), thereby verifying the validity of the 1 : 1 complex stoichiometry as assumed above. Table 1 lists the K_S values and Gibbs free energy ($-\Delta G$) for the inclusion complexes of CDs with LU.

Binding Ability

Many studies have shown that the size and shape-fit concept plays a vital role in the formation of inclusion complexes of host CDs with guest molecules which have various structures. Accordingly, some weak intermolecular forces, such as van der

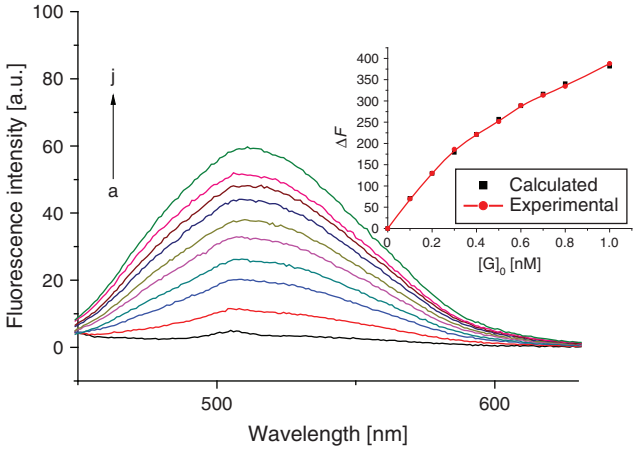

Fig. 2. Fluorescence emission spectra of LU (4.0×10^{-5} M) containing various concentrations of DETA-βCD (from a to k: 0.0×10^{-4}, 0.1×10^{-4}, 0.2×10^{-4}, 0.3×10^{-4}, 0.4×10^{-4}, 0.5×10^{-4}, 0.6×10^{-4}, 0.7×10^{-4}, 0.8×10^{-4}, and 1.0×10^{-4} M of DETA-βCD); λ_{em} 509 nm. The inset shows the non-linear least-squares curve-fitting analysis for the inclusion complexation.

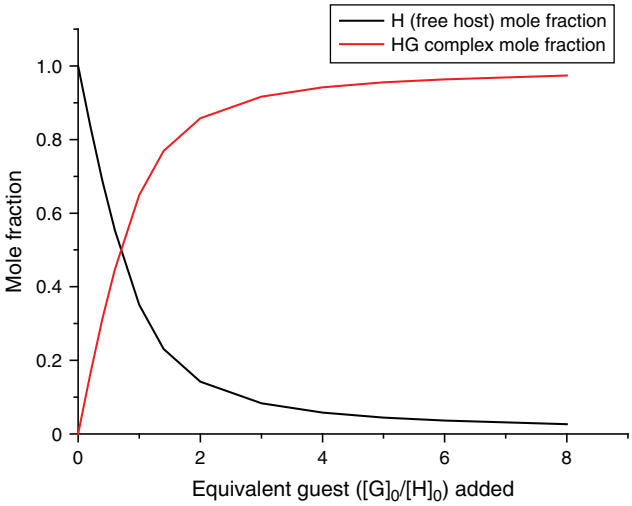

Fig. 3. Speciation plots of the DETA-βCD (red curve) and LU/DETA-βCD complex (black curve) at λ_{UV} 353 nm in pH 7.4 buffer.

Table 1. Stability constant (K_S) and Gibbs free energy change (ΔG) for inclusion complex of host CD with LU (pH 7.4) at 25°C

Host	Guest	K_S [L mol^{-1}]	Log K_S	ΔG [kJ mol^{-1}]
NH_2-βCD	Luteolin	698 ± 30	2.84	−16.24
β-CD		604 ± 20	2.78	−15.88
EN-βCD		1789 ± 80	3.25	−18.56
DETA-βCD		1141 ± 50	3.06	−17.45
TETA-βCD		1117 ± 60	3.04	−17.39

Waals, ion–dipole, hydrogen bond, dipole–dipole, electrostatic, as well as hydrophobic interactions, are accepted for cooperatively contributing to the formation of inclusion complexes. The structural characteristics of β-CD are very important, whereby β-CD has a cyclic truncated-cone cavity with a height of 0.79 nm, an inner diameter of 0.62–0.78 nm, and a cavity volume of 0.262 nm^3.[24] The host–guest size match may dominate the stability of the complexes formed by CDs and LU. From Table 1, the data indicated that the binding constants for the complexes of LU with β-CD, NH_2-βCD, EN-βCD, DETA-βCD, and TETA-βCD decreased in the following order: EN-βCD > TETA-βCD > DETA-βCD > NH_2-βCD > β-CD. By comparing the enhancement effect of some types of CDs for LU, the modified derivatives gave a stronger K_S value than the native β-CD.[25] Furthermore, the number of amino groups may influence the K_S value. In other words, host EN-βCD is more suitable for forming an inclusion complex with LU. The result is understandable because the cavity of β-CD cannot encapsulate LU tightly, and native β-CD can slip onto the guest molecular chain like a bead. Meanwhile the other CDs (NH_2-βCD, DETA-βCD, TETA-βCD) display a lower binding ability than EN-βCD, which has a moderate side chain length. CDs with a longer side chain may increase the level of steric hindrance, thus affecting the entrance of LU in the cavity of the CDs.

Speciation Plot

The speciation plot that shows the molar fraction of CDs formation inclusion complexes was constructed based on the calculated binding constants.[26,27] To understand the conditions and molar proportions, speciation plots revealing the theoretical proportions of CDs and LU/CDs complexes under increasing concentrations of LU were prepared. Fig. 3 shows that when the proportion of the guest/host gradually increased from 0 to 4, the concentration of free DETA-βCDs gradually decreases. However, when the proportion of the guest/host was greater than 4, the concentration of free DETA-βCDs was ~2 %.

^{1}H NMR and 2D NMR analysis

^{1}H NMR spectroscopy is a useful tool to investigate the formation of the LU/CDs. The ^{1}H NMR spectra of LU in the presence of host CDs were compared with the spectrum of LU in order to know the possible inclusion mode of CDs/LU complexes (Fig. 4). The ^{1}H resonances of β-CD, NH_2-βCD, EN-βCD, DETA-βCD, and TETA-βCD were studied in accordance with the reported method.[28,29] Because of its poor water solubility, LU is transparent to ^{1}H NMR under most conditions when D_2O is used as the solvent. Assessment of the LU/CDs complexes by ^{1}H NMR perfectly demonstrated the presence of the framework protons of LU molecule and implied a significant solubility increase for LU/CDs relative to that of native LU (Fig. 4). The chemical shifts of LU or CDs in the ^{1}H NMR spectra can prove the formation of inclusion complexes. Specifically, LU protons (4H) displayed chemical shifts at δ 6.0–7.5 ppm, which were different from the CD protons (usually observed at δ 3.0–5.0 ppm). After formation of the inclusion complex with LU, the H-3 proton of EN-βCD shifted to 0.030 ppm, and the H-5 proton of EN-βCD shifted to 0.049 ppm. The detailed changes in the hydrogen chemical shift values of LU and EN-βCD before and after formation of the inclusion complexes are presented in Table 2. The shift in the proton signals of LU upon addition of CDs shows that the B-ring and C-ring are included in the cavity. Both H-3 and H-5 protons are located in the interior of the CD cavity, and the H-3 protons are located near the wide side of cavity, whereas H-5 protons are located near the narrow side. This result could indicate that LU should be included in the EN-βCD cavity from the narrow side.

It is known that 2D NMR spectroscopy is a powerful tool for studying inter- and intramolecular interactions. Two protons,

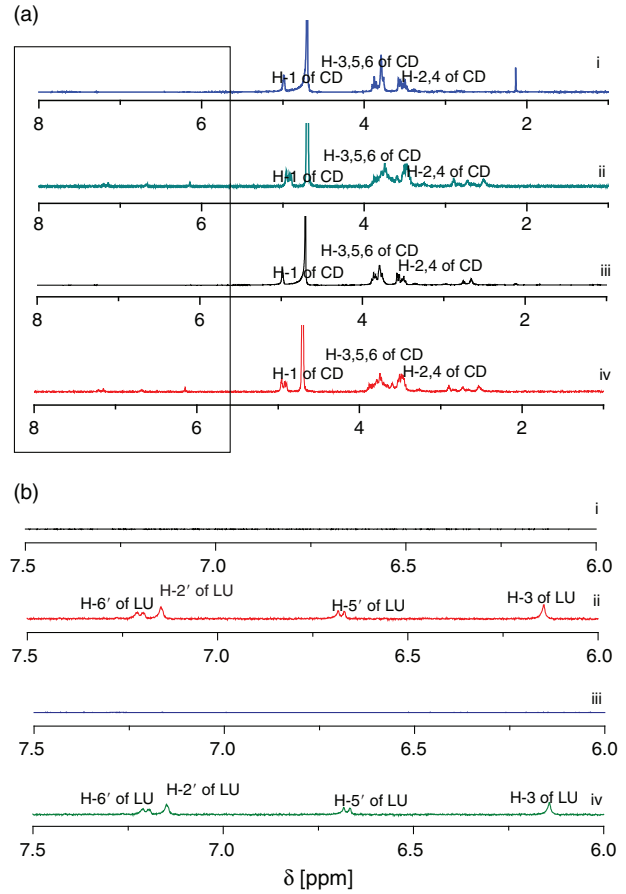

Fig. 4. (a) Complete and (b) enlarged (δ ~6.0–7.7 ppm) ^{1}H NMR spectra of LU in the absence and presence of NH_2-βCD and DETA-βCD measured in D_2O at 25°C: NH_2-βCD (curve i), LU/NH_2-βCD complex (curve ii), DETA-βCD (curve iii), and LU/DETA-βCD complex (curve iv).

when closely located in space, can generate a NOE cross-correlation between the relevant protons in the NOESY or ROESY spectrum. The presence of NOE cross-peaks between protons of two species indicates spatial contacts within 0.4 nm.[30] To obtain more conformation information, we performed 2D ROESY analysis of the inclusion complexes of LU with CDs. The ROESY spectrum of the LU/EN-βCD complex (Fig. 5) showed obvious correlation between LU protons and EN-βCD protons. The results indicated that not only the B-ring, but also part of the C-ring of LU were included in the EN-βCD cavity in accordance with the above results. Based on these results together with the 1 : 1 inclusion stoichiometry deduced by Job plot, the possible inclusion modes of LU with EN-βCD are illustrated in Fig. 6.

X-Ray Diffraction (XRD) Analysis

The XRD patterns of LU, DETA-βCD, their physical mixture, and inclusion complex are illustrated in Fig. 7. LU (spectrum a) was in a crystalline form, but DETA-βCD (spectrum b) is amorphous. The XRD patterns of the physical mixture (spectrum c) confirmed the presence of both species as isolated as the diffractogram showed both LU peaks and amorphous peak features of DETA-βCD. In contrast, the XRD pattern of the LU/DETA-βCD complex (spectrum d) displayed amorphous features and showed halo patterns, and was quite different from

Table 2. Chemical shifts of EN-βCD and LU/EN-βCD complex in D_2O and LU in DMSO at 25°C

		δδ [ppm]		
		LU	EN-βCD	EN-βCD complex
H-1 of EN-βCD	d		4.919	4.945
H-2 of EN-βCD	dd		3.695	3.654
H-3 of EN-βCD	dd		3.830	3.860
H-4 of EN-βCD	dd		3.506	3.503
H-5 of EN-βCD	m		3.783	3.734
H-6 of EN-βCD	dd		3.807	3.748
H-2′ of LU	s	7.395		7.148
H-3 of LU	s	6.894		6.696
H-5′ of LU	d	6.442		6.176
H-6′ of LU	d	7.406		7.207

the superimposition of the patterns of LU and the LU/DETA-βCD physical mixture. The results confirm that the formation of an inclusion complex between CD and LU.

Differential Scanning Calorimetry Analysis

The DSC curves of LU, CDs, physical mixture, and inclusion complex are illustrated in Fig. 8. As observed, LU displayed a sharp endothermic peak at 335°C. DETA-βCD showed two broad and shallow endothermic bands between 80 and 315°C. The DSC curve of the physical mixture was mainly a combination of the curves of the two elements, whereby the LU peaks were only weakly observed for the lower proportions. The curve of the inclusion complex mainly showed features of the DETA-βCD curve. In other words, the characteristic endothermic peaks of LU disappeared, suggesting that an inclusion complex was formed. Similarly, the characteristic DSC peaks of the guest molecules were no longer observed.[31]

Scanning Electron Microscopy (SEM) Analysis

From the SEM analysis (Fig. 9), LU (Fig. 9a) aggregated as ball-like crystals. DETA-βCD (Fig. 9b) is observed as irregularly 3D-shaped crystals. The physical mixture (Fig. 9c) showed characteristics of LU and the 3D-shaped crystals of DETA-βCD coincidently and separately. In contrast, a drastic change in the morphology and shape of the particles was observed in LU/DETA-βCD inclusion complex (Fig. 9d). These images proved that when the powders of LU and CDs were simply mixed together, and existed in their original individuals forms (i.e. when the solutions of the two compounds were mixed), they formed a close association, revealing an apparent interaction in the solid state.

Water Solubility

The inclusion complexes were clearly more soluble than native LU (~0.93 μg mL^{-1}).[32] The solubility remarkably increased to 0.363, 0.705, 0.990, and 1.192 mg mL^{-1} upon the solubilising effects of NH_2-βCD, EN-βCD, DETA-βCD, and TETA-βCD, respectively. In the control experiment, a clear solution was obtained after dissolving LU/NH_2-βCD (28.9 mg mL^{-1}), EN-βCD (33.5 mg mL^{-1}), DETA-βCD (75.6 mg mL^{-1}), TETA-βCD (94.3 mg mL^{-1}), equivalent to 0.363, 0.705, 0.990, 1.192 mg of LU, respectively, in 1 mL of H_2O at room temperature. The result confirmed the reliability of the obtained satisfactory water solubility of the inclusion complexes. However, the solubility of β-CD and SBE-βCD only increased to 1.51- and 15.53-fold.[33] The satisfactory water solubility of LU

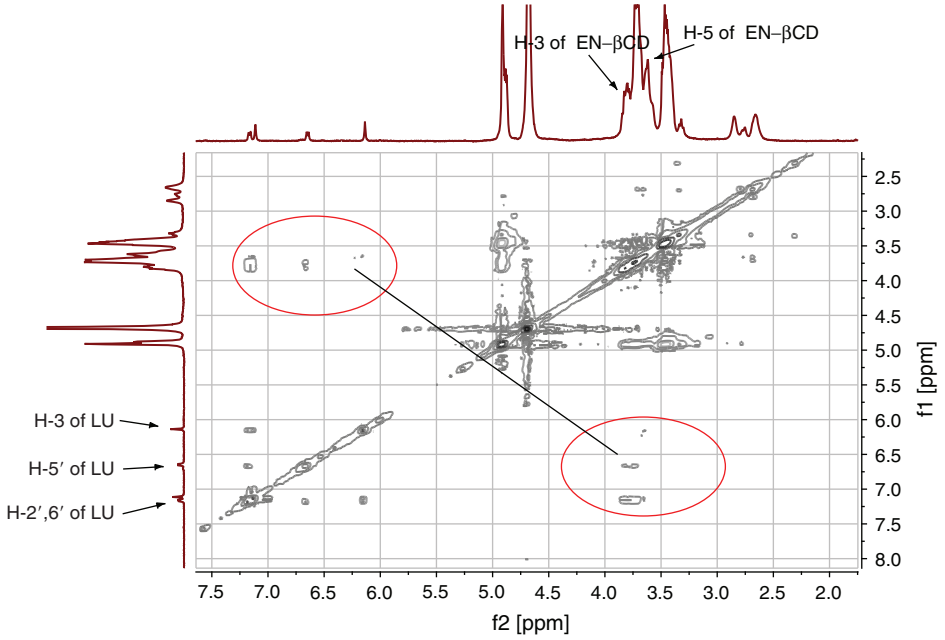

Fig. 5. ROESY spectrum of LU/EN-βCD complex in D_2O.

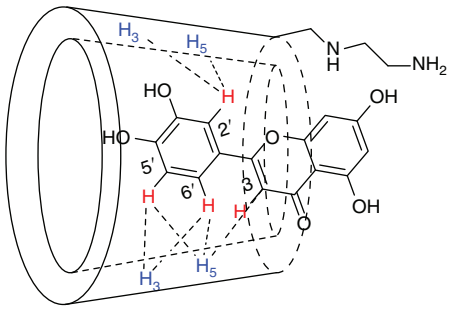

Fig. 6. Possible inclusion mode of EN-βCD/LU complex.

with polyamine-β-cyclodextrin will be beneficial to the medical application of this compound.

Anti-oxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a free radical, stable at room temperature, which presents a violet solution in ethanol. And it is reduced in the presence of an anti-oxidant material. The use of DPPH provides an easy method to evaluate anti-oxidants. We investigated the scavenging ability of LU with DPPH in the presence and absence of CDs. When CD only is mixed with DPPH, the absorbance did not change. In other words, CD had no effect on DPPH. The results are expressed as remaining DPPH (%) as a function of time (Fig. 10). By measuring the activity of LU and its complexes by spectrophotometry, the results clearly showed that the LU complexes displayed higher anti-oxidant activity than the free form of LU. This enhancement in the anti-oxidant activity could be due to stabilisation of the radical in the cyclodextrin cavity. In other words, in the presence of CDs, LU radical is more stable, probably due to the hindered oxidation of the apolar cavity of CD.

Cytotoxic Activity

The cytotoxicity of LU and the four LU/CD complexes (NH_2-βCD, EN-βCD, DETA-βCD, TETA-βCD) were studied using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. The IC_{50} (half-maximal inhibitory concentration) values have been calculated and presented in Table 3. These complexes revealed a satisfactory anti-proliferative activity when compared with native LU. The IC_{50} values of the four complexes and LU were 10.36, 8.64, 4.21, 7.65, and 43.25 μM, respectively in Hep G_2 cell line. In the control tests, the IC_{50} values of NH_2-βCD, EN-βCD, DETA-βCD, and TETA-βCD were tested following addition (5 mM) to Hep G_2 cell line. The results indicated that the derivatives of β-CD were non-toxic. The increase in cytotoxicity could be due to the formation of the inclusion complexes. The inclusion of LU into CDs can increase the transport of LU through the cellular membranes, thus improving its cytotoxicity activity.[34]

Conclusion

In this study, the inclusion complex of LU with polyamine-β-cyclodextrin was characterised by its binding behaviour, binding ability, speciation plot, solubility, and anti-oxidant activity and anti-cancer activity. The results indicated that polyamine-modified β-CDs could improve the water solubility of LU significantly. EN-βCD was the most promising host for inclusion complex of LU. The anti-oxidant studies showed that the inclusion complexes had better anti-oxidant activities than free LU. In vitro cytotoxicity studies showed that the inclusion complex had much higher anti-proliferative activities than native LU. Thus, this type of inclusion complex is expected to represent a significant step in the design of a novel formulation of LU for application in herbal medicine and natural health products.

Experimental

Materials

Luteolin (3′,4′,5,7,-tetrahydroxyflavone, >98 %) was purchased from Nanjing Jingzhu Bio-technology Co., Ltd. Modified CDs, NH_2-βCD, EN-CD, DETA-βCD, and TETA-βCD, were synthesised in accordance with reported procedure.[35]

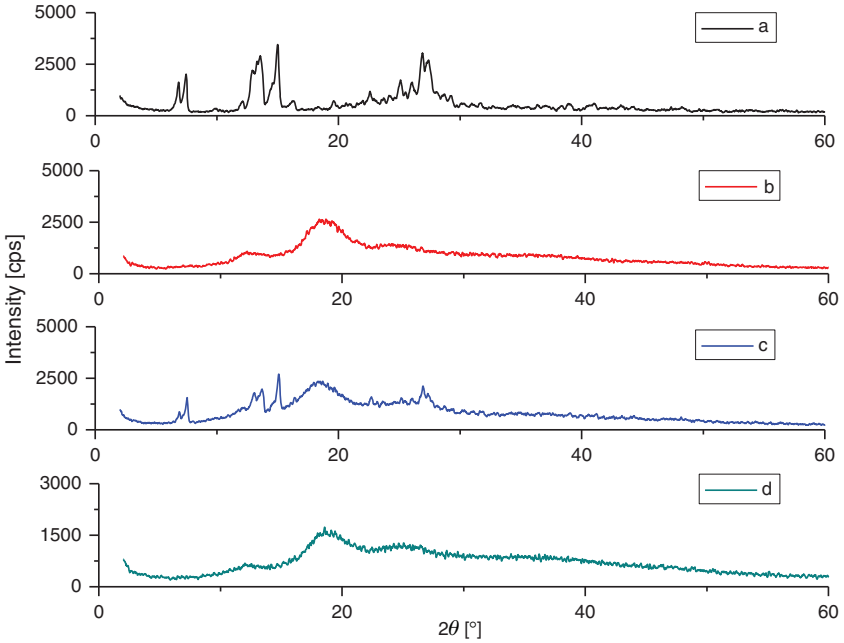

Fig. 7. XRD patterns of (a) LU, (b) DETA-βCD, (c) LU/DETA-βCD physical mixture (1 : 1), and (d) LU/DETA-βCD inclusion complex.

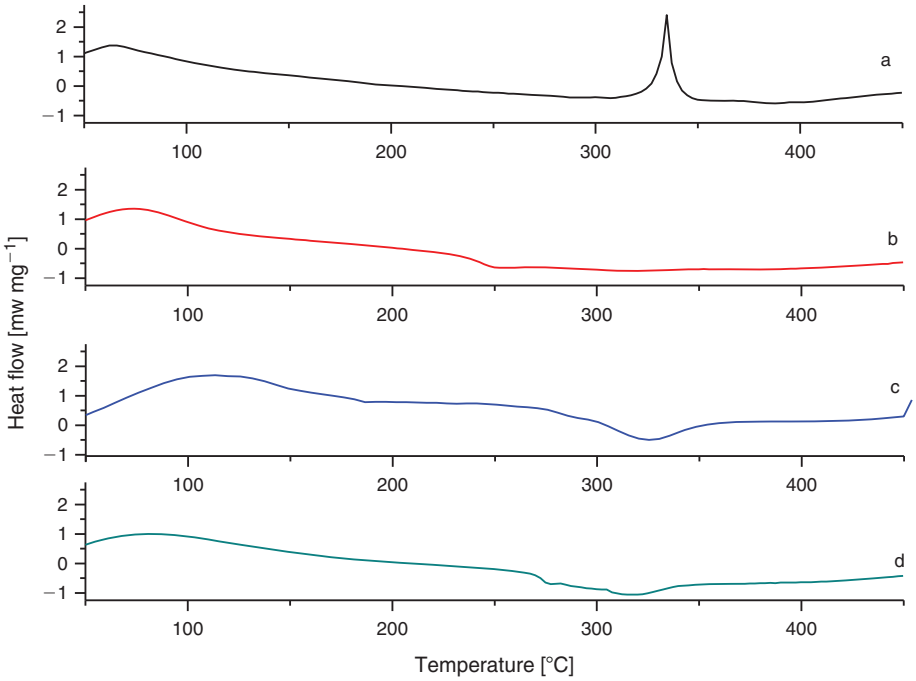

Fig. 8. DSC patterns of (a) Lu, (b) DETA-βCD, (c) LU/TETA-βCD physical mixture (1 : 1), and (d) LU/TETA-βCD inclusion complex.

β-CD was purchased from MengZhou Huaxin Biological Technology Co., Ltd (Shanghai, China). DPPH was purchased from Sigma. Other chemicals and reagents were of analytical grade. Double-distilled and deionised water was used.

Preparation of LU/NH_2-βCD, LU/EN-βCD, LU/DETA-βCD, LU/TETA-βCD Inclusion Complexes

LU (0.03 mM) and the required CD (0.01 mM) were added to distilled water in a round flask, and then stirred for 4 days at room temperature in the dark. The precipitate was removed by filtration using a 0.45 μm Millipore membrane. Then, the filtrate was evaporated under reduced pressure to remove the solvent and dried under vacuum to obtain the LU/CD complexes.

Preparation of LU and NH_2-βCD, EN-βCD, DETA-βCD, TETA-βCD Physical Mixtures

The physical mixture was made by grinding together a 1 : 1 molar mixture of LU and CD in an agate mortar for 15 min.

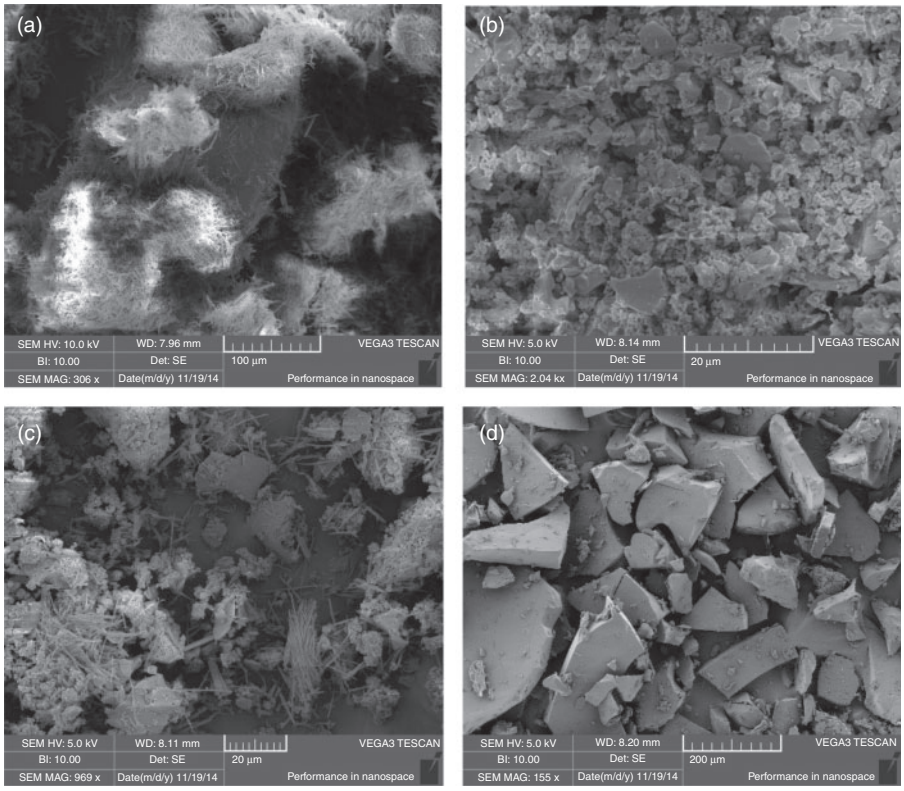

Fig. 9. EMS images of (a) LU, (b) TETA-βCD, (c) physical mixture of TETA-βCD and LU, and (d) LU/TETA-βCD inclusion complex.

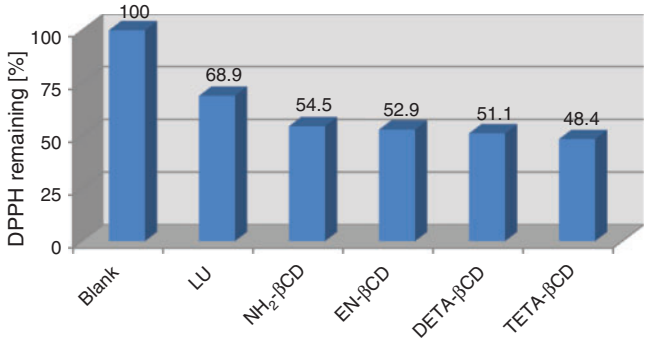

Fig. 10. Percentage content of DPPH remaining for samples blank, free LU, and LU in the presence of NH_2-βCD, EN-βCD, DETA-βCD, and TETA-βCD.

Table 3. IC_{50} of LU and LU/CD complexes in human hepatoma cell line Hep G_2

Sample	IC_{50} [μM][A]
LU	43.25
NH_2-CD/LU	10.36
EN-CD/LU	8.64
DETA-CD/LU	4.21
TETA-CD/LU	7.65

[A]The concentrations of free LU, physical mixtures, and inclusion complexes mentioned in this table are expressed as per mole of LU.

Stoichiometry

The stoichiometry of the inclusion complexes of LU with CDs was measured by Job's methods.[36] The Job plot was determined using fluorescence spectroscopy conducted in a pH 7.4 buffer solution. The total molar concentration (i.e. the combined concentration of LU and CD was kept constant (4.0×10^{-5} M), and the molar ratio of LU (i.e. [LU]/([LU] + [CD]) was varied from 0.1 to 0.9.

Spectral Titration

The spectral titration studies were conducted using CD (2.0×10^{-3} M) and LU (4.0×10^{-4} M) solution in a KH_2PO_4–NaOH (pH 7.4) buffer solution. In a 10 mL colorimetric tube, 1.0 mL of 4.0×10^{-4} M LU and varied amounts of 2.0×10^{-3} M CD (β-CD, NH_2-βCD, EN-βCD: 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mL; DETA-βCD, or TETA-βCD: 0.0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4, 2.7 mL) were added sequentially. The mixed solution was diluted to the mark with buffer, and then ultrasonically oscillated for 30 min at 30°C, after which the fluorescence spectra were measured at $\lambda_{ex}/\lambda_{em} = 420$ nm/510 nm (λ_{ex} = excitation wavelength; λ_{em} = emission wavelength).

Speciation Plot

The speciation plots were obtained using CD (4.0×10^{-4} M) and LU (4.0×10^{-4} M) solution in a KH_2PO_4–NaOH (pH = 7.4) buffer solution. In a 10 mL colourimetric tube, 0.5 mL CD and varied amounts of LU (0.0, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 mL) were added sequentially. The mixed solution was diluted to the mark with buffer, then ultrasonically oscillated for 30 min at 30°C, after which the ultraviolet spectra were measured at 353 nm. The speciation plot showing the molar fraction of LU bound to the CDs at steady-state was calculated according to the website *www.supramolecular.org.*

^1H NMR and 2D NMR

^{1}H NMR spectra of the LU/CD inclusion complexes and LU monomer were obtained using a Bruker Avance DRX spectrometer at 500 MHz and 298 K using D_2O and [D6]DMSO, respectively. Data were collected without an external reference in order to avoid potential interactions with the CDs. ROESY data were obtained on a Bruker Avance DRX500 instrument and were implemented in D_2O.

XRD

The XRD patterns were recorded on a PHI 5000 VersaProbe II using Cu-Kα radiation (λ 1.5460 Å, 40 kV, 100 mA) at room temperature, and a scanning speed was 4° min^{-1}. Powder samples were mounted on a vitreous sample holder and scanned with a step size of 2θ 0.02° in the 2θ range of 5–65°.

DSC

DSC measurements were performed on a NETZSCH STA449F3, using a heating rate of 10°C min^{-1} in the temperature range of 30–460°C in a dynamic nitrogen atmosphere (flow rate = 100 mL min^{-1}).

SEM Analysis

SEM analysis was carried out on a Jeol JSM-840 scanning electron microscope. Before examination, the samples were gold sputter-coated to improve their electrical conductivity.

Solubilisation Test

The water solubility of the LU/CD complexes was assessed by preparation of their saturated solutions.[37] An excess amount of complex was placed in 2 mL of water, and the mixture was shaken in a water bath shaker for 2.5 h at 25 ± 0.5°C. Then, the solution was filtered using a 0.45 µm Millipore membrane. The filtrate was then evaporated under reduced pressure to dryness, and the resulting solid weighed.

DPPH Scavenging Capacity Assay

The ability of the LU/CDs to scavenge DPPH radicals was evaluated according to the procedure described by Mensor et al.[38] A volume of 50 µL of 50 µg mL^{-1} sample was mixed with 150 µL of 100 µg mL^{-1} DPPH ethanol solution. The mixture reacted for 30 min at room temperature in the dark. The absorbance was measured at 518 nm, using a spectrophotometer, and converted into the percentage anti-oxidant activity using Eqn 4.[39] All experiments were performed in triplicate.

$$\%\text{DPPH remaining} = (A_{A(t)}/Ac_{(0)}) \times 100\,\% \qquad (4)$$

where $Ac_{(0)}$ = initial absorbance of DPPH and $A_{A(t)}$ = final absorbance of DPPH.

Cell Line and Culture Medium

Human hepatoma cell line (Hep G_2) is one of the most common experimental models used in in vitro studies.[40] Thus, we cultured Hep G_2 cells at 4×10^4 mL^{-1} in RPMI-1640 medium containing 10 % heat-inactivated fetal bovine serum supplement for 24 h incubation at 37°C under 5 % CD_2 in air. Cells were seeded at 4×10^4 mL^{-1} and treated with the indicated amounts of LU and its inclusion complex. First, cell viability was evaluated by a microculture tetrazolium reduction assay using MTT. Next, 50 µL of MTT stock solution, at a concentration of 4 mg mL^{-1} in PBS was added to 150 µL of the cell cultures in the 96-microwell flat-bottom plate for 24 h incubation at 37°C. Then, the solutions in the plates were centrifuged (1000 *g* for 5 min.), and the MTT-containing culture medium was removed. The precipitated formazan was dissolved in 120 µL DMSO. Results were read within 15 min using a spectrometer at 490 nm. Finally, the means of the triplicates were calculated. Cell inhibition rate was expressed as a percentage of the control samples.

Supplementary Material

Stoichiometry and speciation plots; absorption, ^{1}H NMR and ROESY spectra, and SEM images for various inclusion complexes are available on the Journal's website.

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