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A structure-differential binding method for elucidating the interactions between flavonoids and cytochrome-c by ESI-MS and molecular docking

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ABSTRACT

The study of noncovalent interactions between pharmaceutical molecules and proteins is essential for understanding molecular mechanisms of protein function, and provides foundations for *de novo* therapeutic agent design. Electrospray ionization mass spectrometry (ESI-MS) has nowadays become a popular tool for analyzing the noncovalent protein complexes, however it usually has difficulty in determining the interaction sites and binding mechanisms. In this work, a new structure-differential binding (SDB) method, combined with ESI-MS and molecular docking (MD) techniques (SDB–ESIMS–MD), was developed and applied to a study of the binding interactions in noncovalent protein–small drug molecule complexes for the characterization of binding sites and binding modes. Using this developed method, protein complexes of flavonoid and flavonoid glycoside ligands and cytochrome-c (Cyt-c) were studied in detail. ESI-MS was used to determine the relative binding affinities and dissociation constants of flavonoid–Cyt-c complexes, and to measure the changes in the stability of the protein complexes with the structural modifications of the ligands for identifying effective binding functional groups. Molecular docking simulations complemented ESI-MS experiments by providing the protein–ligand interaction profile of each complex and displaying the binding mode for each interaction. This SDB–ESIMS–MD method can be applied to a broad range of protein–drug interactions and used to guide further research in the study of structure–binding relationship between drug molecules and targeted biomacromolecules.

1. Introduction

Noncovalent interactions between pharmaceutical molecules and proteins play important roles in many cellular processes, drug metabolisms and developments of novel therapeutic agents [1–3]. Elucidating binding interactions, structures and stability of noncovalent protein complexes is essential for the understanding of biological processes. There are numerous methods for studying interactions between drugs and proteins, such as UV–vis absorption, fluorescence, circular dichroism and NMR spectroscopy [4–7]. In the last two decades, electrospray ionization mass spectrometry (ESI-MS) has become a popular tool for studying noncovalent interactions in solution, because ESI is a "soft" ionization technique that can ionize molecules directly from aqueous-based solutions designed to mimic physiological conditions [8–14]. ESI gently evaporates the solvent, leaving solution-based macromolecular complexes intact while transforming them into gas-phase ions. Even weakly-bound complexes with millimolar dissociation constants can often survive the ESI process [15–17].

However, ESI-MS analysis of protein complexes usually provides little information on their interaction sites and binding mechanisms. Identification of binding sites and binding modes within a protein complex is important to understand molecular mechanisms of protein function, and also provides a foundation for *de novo* drug design, structural identification and comparison of functional sites. In this work, we proposed a novel structure-differential binding method in combination with ESI-MS and molecular docking (SDB–ESIMS–MD) techniques to identify effective binding sites and characterize the interactions of protein–ligand complexes. In order to explore the structural unit or functional groups in drug ligands that play important roles in the binding, a series of structure-specific drug molecules were selected as model ligands to bind with the targeted protein. These ligands were carefully selected that they differ from one another by only eliminating a structural unit or modifying one functional group. The SDB method is illustrated in Scheme 1. The binding constants and binding affinities of these SDB ligands bound to the protein were determined by direct ESI-MS assay and competition ESI-MS

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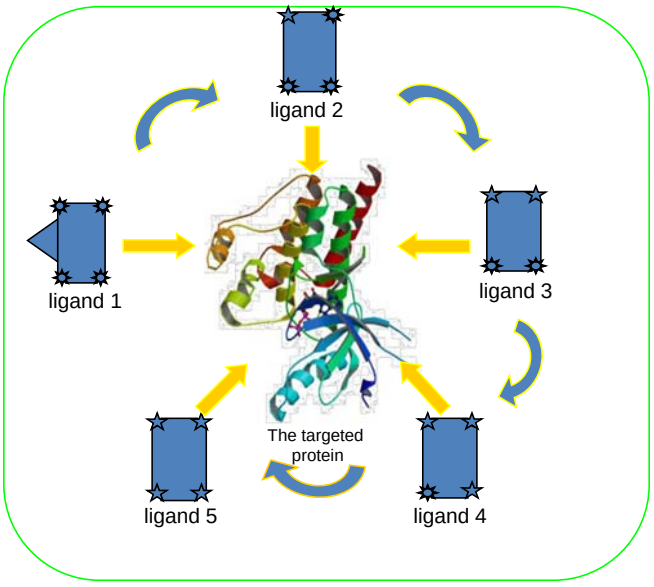

Scheme 1. Schematic diagram of the structure-differential binding (SDB) method.

experiments [18–21]. The trend of these bindings revealed how the structural differences in ligands affect the stability of the protein complex and thus probed the preferred binding sites and binding modes. Computational MD simulations complemented ESI-MS experiments by providing insights into binding profiles, interaction strength and forces for these noncovalent protein complexes.

Flavonoids and flavonoid glycosides have a wide range of biological effects *in vitro*, including antioxidizing, anti-inflammatory and anti-carcinogenic, and so have been investigated for their potential health benefits in humans [22–31]. A flavonoid glycoside molecule consists of a common flavonoid linked to different sugars. Preliminary studies have shown that flavonoid compounds could induce mechanisms that may kill cancer cells and inhibit tumor invasion. Cytochrome-c (Cyt-c), used as a model protein in this study, is an electron-carrying protein found in mitochondria of all aerobic organisms [32] and it also plays roles in energy metabolism and cell apoptosis [33,34]. It can also serve as a prognostic marker during cancer therapy [35]. Cyt-c is a well-characterized protein because of its small size (12.36 kDa) and relative ease of ionization [36]. We have observed that Cyt-c has a higher binding affinity to flavonoids and flavonoid glycosides than other standard proteins such as myoglobin and hemoglobin. We herein have applied the SDB–ESIMS–MD method to study the interactions between Cyt-c and a series of structurally-different flavonoid glycoside and flavonoid molecules and to characterize the effective binding sites and binding mode of the protein complexes. The selected ligands were tectoridin (Tec), hesperidin (Hes), daidzin (Din), daidzein (Dein), 7-methoxy-4′-hydroxyisoflavone (4H7M), 4′-methoxy-7-hydroxyisoflavone (4M7H) and 4′,7-dimethoxyisoflavone (4M7M). Their molecular structures are shown in Fig. 1. Tec, Hes and Din are flavonoid glycosides. Dein (4′-hydroxy-7-hydroxyisoflavone) is the isoflavone that is composed of Din with a 7-O–glucose. Dein becomes 4M7H by the replacement of the 4′-OH with a 4′-OCH_3. Similarly, 4M7H becomes 4H7M by the replacement of the 7-OH with a 7-OCH_3. When both of the C4′ and C7 –OH groups in Din are substituted by two –OCH_3, it produces 4M7M.

The results of this study have provided the first detailed qualitative and quantitative description of the binding interactions within Cyt-c–flavonoid/flavonoid–glycoside complexes. A comparison of the stabilities measured by ESI-MS for the structure-differential protein complexes, together with the interaction profiles given by MD, allowed the preferred binding sites and intermolecular hydrogen bonds (H-bonds) in the protein complexes to be identified. This SDB–ESIMS–MD method therefore overcomes the shortfall of conventional ESI-MS that has difficulty in determining the binding sites and interaction modes, and thus significantly adds to the variety of mass spectrometry-based techniques for the study of noncovalent protein complexes.

2. Experimental

2.1. Materials and sample preparation

Cytochrome-c from horse heart was obtained commercially as lyophilized powder from Sigma Chemical Co. (St Louis, MO, USA) and was used without further purification. Tectoridin (purity≥98%), hesperidin (purity≥98%), daidzin (purity≥98%), daidzein (purity≥98%) and 4′-methoxy-7-hydroxyisoflavone (purity≥98%) were obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China). 7-methoxy-4′-hydroxyisoflavone (purity≥97%) was obtained from Shunbo Biotech Co. Ltd (Shanghai, China). 4′,7-dimethoxyisoflavone (purity≥98%) was obtained from Alfa Aesar Co. Ltd (Tianjin, China). HPLC-grade methanol was purchased from Fisher Scientific (Waltham, MA, USA). Glacial acetic acid was purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). The water purification system was obtained from Mole Scientific Instrument Co. Ltd (Shanghai. China). Flavonoid ligands were dissolved in methanol and then diluted with purified water to prepare the stock solutions of 250 μM. Cyt-c was mixed with appropriate amounts of stock solutions of ligands in 10 mM ammonium acetate (at a pH of 6.8) to a final concentration of 0.5 μM, and with different concentrations of ligands at 2.5 μM, 4 μM, 5 μM, 6 μM and 7.5 μM, respectively (giving the molar concentration ratios of 1:5, 1:8, 1:10, 1:12 and 1:15). Each mixture solution was allowed to equilibrate at room temperature for 1 h before ESI-MS analysis. Cyt-c and two equimolar ligands at 1:5:5 M ratios were used in the competitive binding experiments and control experiments.

2.2. Electrospray ionization mass spectrometry

All mass spectra were acquired from an Agilent 6520 quadrupole-time of flight (Q-TOF) mass spectrometer with a standard ESI source in the positive ion mode. The samples were injected via a syringe pump and were introduced into the ESI source at a flow rate of 6 μL min^{-1}.

The Tec–Cyt-c complex served as a model ligand-bound Cyt-c complex for establishing appropriate instrumental conditions for ESI-MS binding measurements. The ESI source parameters were optimized in a stepwise fashion to achieve a stable complex signal and to minimize gas-phase decomposition of noncovalent complexes. The gas temperature was 130 °C, the flow of drying gas was 8 L min^{-1}, and the nebulizer was at 15 psi. The capillary voltage was 4500 V and the fragmentor was 175 V. All samples were prepared in triplicate and analyzed by ESI-MS. Each ESI-MS experiment was repeated three times and all the ESI-MS spectra were obtained under the same experimental conditions. Data were acquired by an Agilent Masshunter workstation, and the deconvoluted mass spectra were obtained by Agilent Masshunter Qualitative Analysis.

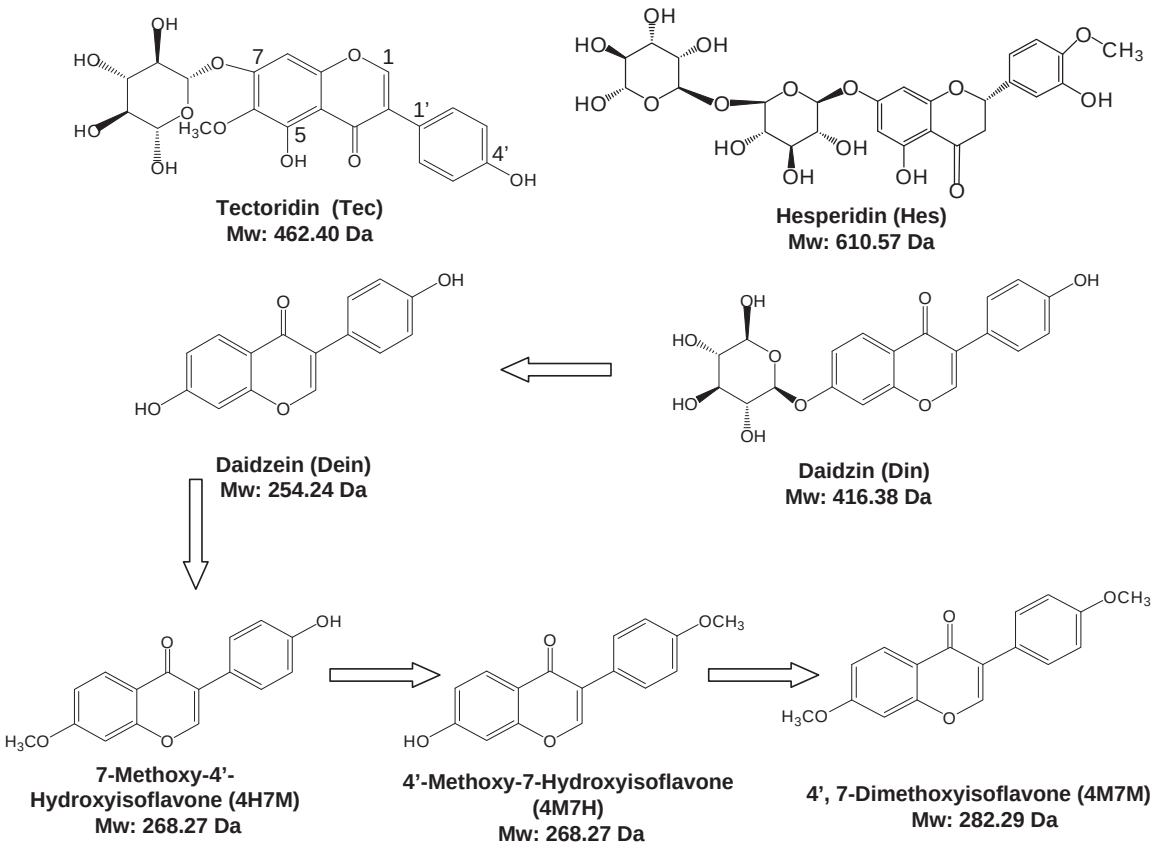

Fig. 1. Molecular structures of flavonoid glycosides and structure-differential flavonoids used to bind with cytochrome-c.

2.3. Molecular docking

The crystal structure of Cyt-c was obtained from the protein data bank (PDB). The 3D structures of the ligands (mol 2) were downloaded from the website http://zinc.docking.org/. IGEMDOCK software was used for molecular docking. The parameters used in this computational modeling include population size ($N=200$), generations ($N=80$) and number of solutions ($N=10$). For each docking, ligand optimization was stopped when either the convergence was below a certain threshold value or the iterations exceeded the maximal preset value of 80. The "best docked" files were chosen for analysis and figures were visualized by Raswin and ChemDraw 3D.

3. Results and discussion

3.1. Analysis of the noncovalent complexes of flavonoid glycosides and cytochrome-c by ESI-MS

Fig. 2A shows the ESI mass spectrum of aqueous solution of Cyt-c (0.50 μM) and tectoridin (Tec, 2.50 μM). The mass spectrum displays abundant signals for the Cyt-c ions and the Tec-bound complexes with a charge distribution from 8+ to 13+. The deconvoluted mass spectrum gives the binding stoichiometry of the noncovalent complexes. The 1:1 and 1:2 protein–ligand complexes were consistently detected with comparable relative abundance from the solutions at different Tec–Cyt-c concentration ratios. The detected molecular mass of cytochrome-c from equine heart was 12,359.40 ± 0.12 Da, and the 1:1 and 1:2 Tec–Cyt-c complexes gave the peaks of 12,821.8 ± 0.50 Da and 13,284.2 ± 0.6 Da, respectively, in the deconvoluted mass spectrum.

In addition, we have observed the formation of a Cyt-c-bound complex with the other two flavonoid glycosides, namely hesperidin (Hes) and daidzin (Din). The ESI and deconvoluted mass spectra for each complex are shown in Fig. 2B and C. Ions corresponding to free Cyt-c and noncovalent complexes were observed, predominantly at charge states $n=8+$ to 13+. The deconvoluted mass spectrum confirmed the formation of 1:1 and 1:2 complexes of Cyt-c and ligands. The binding of each Hes molecule to Cyt-c led to a mass increment of 610.57 Da whereas binding of a Din molecule increased the mass by 416.38 Da.

3.2. Characterization of the interactions between SDB flavonoid model molecules and cytochrome-c by ESI-MS

Under the same experimental condition as that for Cyt-c–flavonoid glycosides in Section 3.1, we employed also ESI-MS to analyze binding between Cyt-c and the structure-differential flavonoid model molecules, namely daidzein (Dein), 7-methoxy-4′-hydroxyisoflavone (4H7M), 4′-methoxy-7-hydroxyisoflavone (4M7H) and 4′,7-Dimethoxyisoflavone (4M7M). Their mass spectra are given in Fig. 3A–D. The ESI-MS spectra of these flavonoids with Cyt-c are similar to those of Cyt-c–flavonoid glycoside solutions, as complexes between Cyt-c and Dein, 4H7M or 4M7H were also detected. Complexes between Cyt-c and 4M7M, however, was not observed (see Fig. 3D). From the comparison of these SBD flavonoid ligands, it is presumed that 4M7M cannot bind to Cyt-c because of the lack of –OH in the structure of 4M7M.

In order to further confirm that the six Cyt-c-bound complexes observed in ESI-MS did not originate from nonspecific Cyt-c–ligand interactions during the ESI process, control experiments were carried out by the addition of 4M7M as the reference molecule into each ESI solution of Cyt-c with another ligand, giving the mixture solutions of Cyt-c–Tec–4M7M, Cyt-c–Hes–4M7M, Cyt-c–Din–4M7M, Cyt-c–Dein–4M7M, Cyt-c–4H7M–4M7M and Cyt-c–4M7H–4M7M. The concentration of Cyt-c was

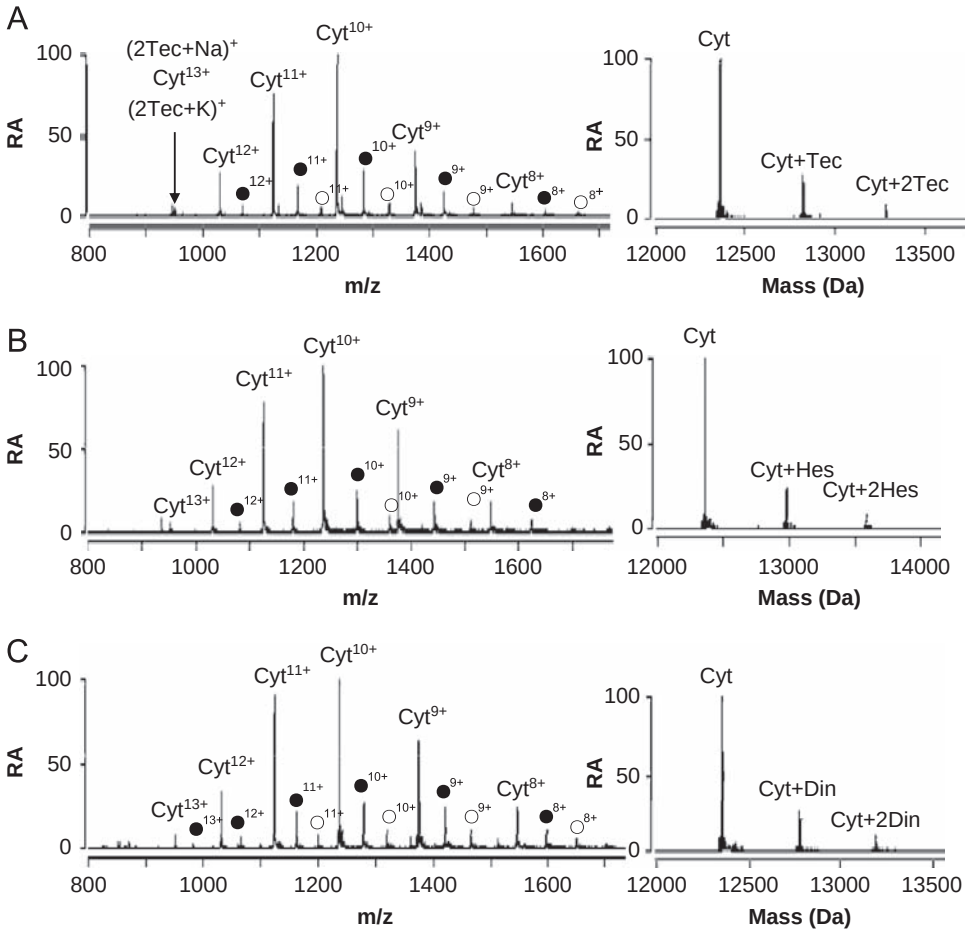

Fig. 2. The multiply charged and deconvoluted ESI-MS spectra of the complexes of cytochrome-c with (A) tectoridin (Tec), (B) hesperidin (Hes) and (C) daidzin (Din) at a 1:5 protein–ligand molecular ratio. The concentration of Cyt-c is 5×10^{-7} mol L^{-1}. • represents the 1:1 complexes; ○ represents the 1:2 complexes.

0.50 μM in the mixture solution; 4M7M and the other ligand were at 2.5 μM, respectively. The control experiments were performed under the same ESI-MS conditions as the above experiments. Cyt-c–Tec, Cyt-c–Hes or Cyt-c–Din complexes at 1:1 and 1:2 stoichiometric ratios were observed but no Cyt-c–4M7M complex were detected in the corresponding ESI-MS. Similar phenomena were observed for the addition of 4M7M to other flavonoid–Cyt-c solutions. Mass spectra of control experiment solutions are given in the Supplementary Information.

3.3. Competition experiments for the determination of relative binding affinities of flavonoid glycoside/flavonoid SDB ligands with cytochrome-c

The binding affinities of six Cyt-c–flavonoid glycosides/flavonoids complexes were investigated by competition experiments, in which the concentration of Cyt-c was fixed at 0.50 μM and the two competing binding ligands at equimolar amount of 2.5 μM. Fig. 4 displays the deconvoluted spectra of Cyt-c with five pairs of ligands, namely Tec and Hes, Hes and Din, Din and Dein, Dein and 4M7H, Dein and 7M4H. The ESI-MS spectra for each mixture solution are also provided in the Supplementary Information. From the relative abundance of the corresponding 1:1 and 1:2 complexes in the mass spectra, we can determine which ligand is more favoured to bind to Cyt-c. The results for the relative binding affinities are as follows: Tec > Hes (Fig. 4A), Hes > Din (Fig. 4B), Din > Dein (Fig. 4C), Dein > 4H7M (Fig. 4D), Dein > 4M7H (Fig. 4E). We did not choose the mixture of 4M7H and 7M4H to perform the competition experiment because these isomers have identical molecular weights, making their noncovalent complexes indistinguishable in the ESI-MS spectra. Comparing Fig. 4D with E, it can be seen that although Cyt-bound Dein is the dominant complex in both spectra, a minor peak corresponding to Cyt-c–4H7M complex ion is still discernible in Fig. 4D but Cyt-c–4M7H complex ion is not present in Fig. 4E. Because all these competition experiments were accomplished under the same experimental conditions, such result indicates that 4H7M has a relatively higher binding affinity than 4M7H. Therefore, the order of the total binding affinities can be summarized as Tec > Hes > Din > Dein > 4H7M > 4M7H > 4M7M.

3.4. Determination of binding constants of the Cyt-c-bound flavonoid glycoside/flavonoid complexes

The binding constants of these Cyt-c-bound flavonoid glycosides/flavonoid complexes have been determined by a direct ESI-MS assay [15,37–40], based on quantification of the relative intensities of the ligand-bound complex and the free protein ions in the ESI mass spectra, namely I_{PLn} and I_{Pn}. Previous ESI-MS studies have shown that when the ligand is small compared to the protein, the size and surface properties of the free and ligand-bound proteins are similar and so their ESI-MS response factors are similar. Thus, the ion relative intensity determined in the gas phase is representative of the equilibrium concentration ratio [39–43]. The measured relative peak intensity ratio, (r), is assumed to be equivalent to the equilibrium concentration ratio of the ligand-bound protein complex and total protein species in

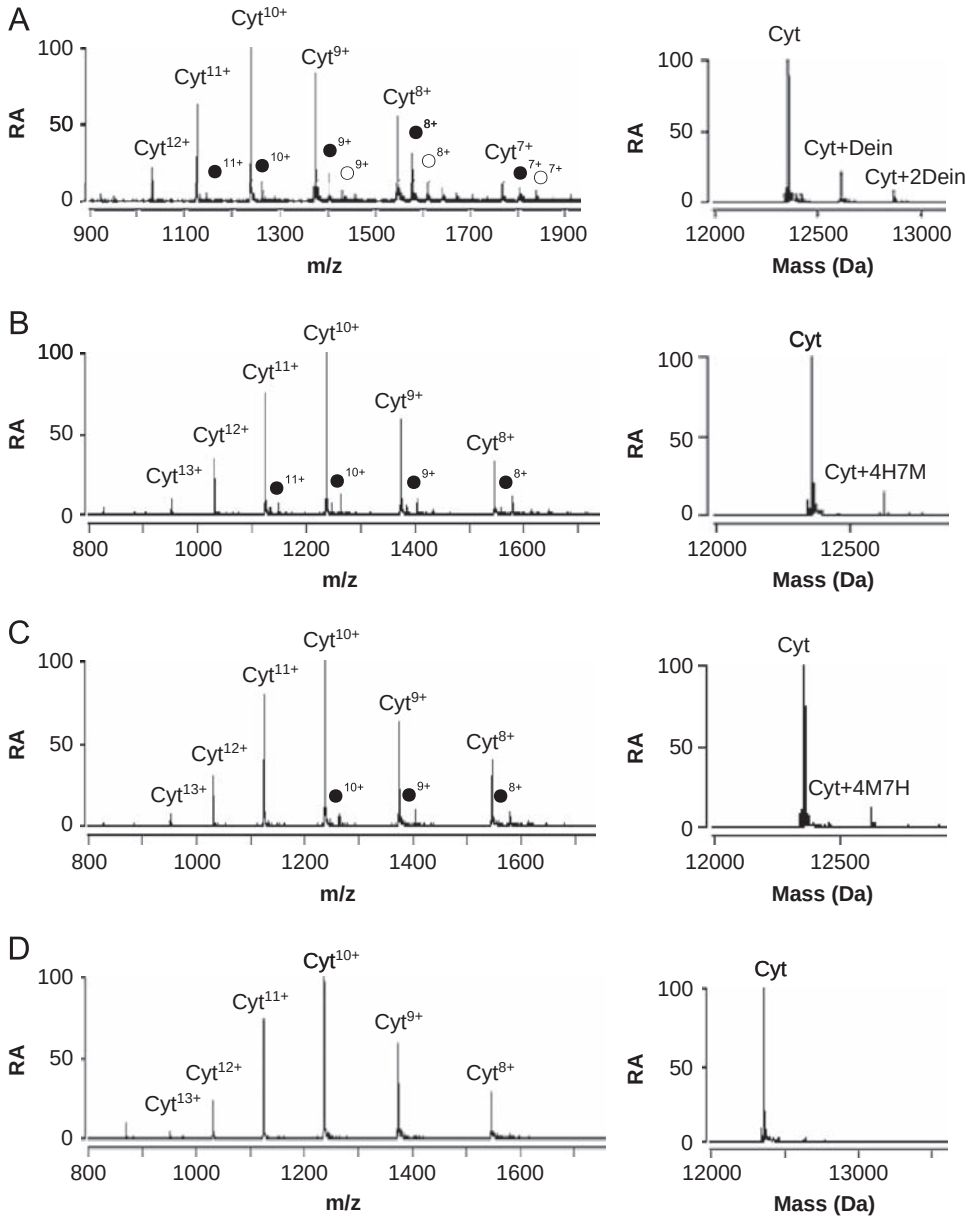

Fig. 3. The multiply charged and deconvoluted ESI-MS spectra of the complexes of cytochrome-c with (A) daidzein (Dein), (B) 7-methoxy-4′-hydroxyisoflavone (4H7M), (C) 4′-methoxy-7-hydroxyisoflavone (4M7H) and (D) 4′,7-dimethoxyisoflavone (4M7M) at a 1:5 protein–ligand molecular ratio. The concentration of Cyt-c is 5×10^{-7} mol L^{-1}. • represents the 1:1 complexes; ○ represents the 1:2 complexes. Protein–ligand complex was not observed in D.

solution, Eqs. (3) and (5).

$$PL_n \overset{Kd_n}{\longleftrightarrow} P + nL \tag{1}$$

$$Kd_n = \frac{[P][L]^n}{[PL_n]} \tag{2}$$

$$r_1 = \frac{[PL_1]}{[P]_0} = \frac{IPL_1}{IP + IPL_1 + IPL_2} \tag{3}$$

$$Kd_1 = \frac{(1-r_1)([L]_0 - r_1[P]_0)}{r_1} \tag{4}$$

$$r_2 = \frac{[PL_2]}{[P]_0} = \frac{IPL_2}{IP + IPL_1 + IPL_2} \tag{5}$$

$$Kd_2 = \frac{(1-r_1-r_2)([L]_0 - r_1[P]_0 - 2r_2[P]_0)^2}{r_2} \tag{6}$$

Based on the above equations, the equilibrium dissociation constants Kd_1 and Kd_2 were calculated for the 1:1 and 1:2 Cyt-c–ligand complexes. These calculated *Kd* values and their relative standard deviations (*RSD*s) at given concentration ratios of Cyt-c and ligands are shown in Table 1.

The *RSD*s are satisfactory for Kd_1 values, being less than 4.4%. The *RSD*s for Kd_2 are higher than those for Kd_1, because the corresponding 1:2 complexes have lower relative abundances on the ESI-MS spectra. The order of Kd_1 for the Cyt-c-bound complexes is Tec < Hes < Din, Dein < 4H7M < 4M7H. Among the studied Cyt-c-bound complexes, Cyt-c–Tec complex has the lowest Kd_1 values whereas Cyt-c–4M7H has the highest, the difference being about 6-fold. Because the abundances of the 1:2 Cyt-c–4H7M and Cyt-c–4M7H complexes are too weak to be measured accurately from the ESI-MS spectra, their Kd_2 were not reported. The order of Kd_2 is Hes≤Tec < Din < Dein. Unlike the Kd_1 values, the Kd_2 of Cyt-c–Hes is slightly lower than that of Cyt-c–Tec. The other Kd_2 values follow the same trend as the Kd_1 values. Since

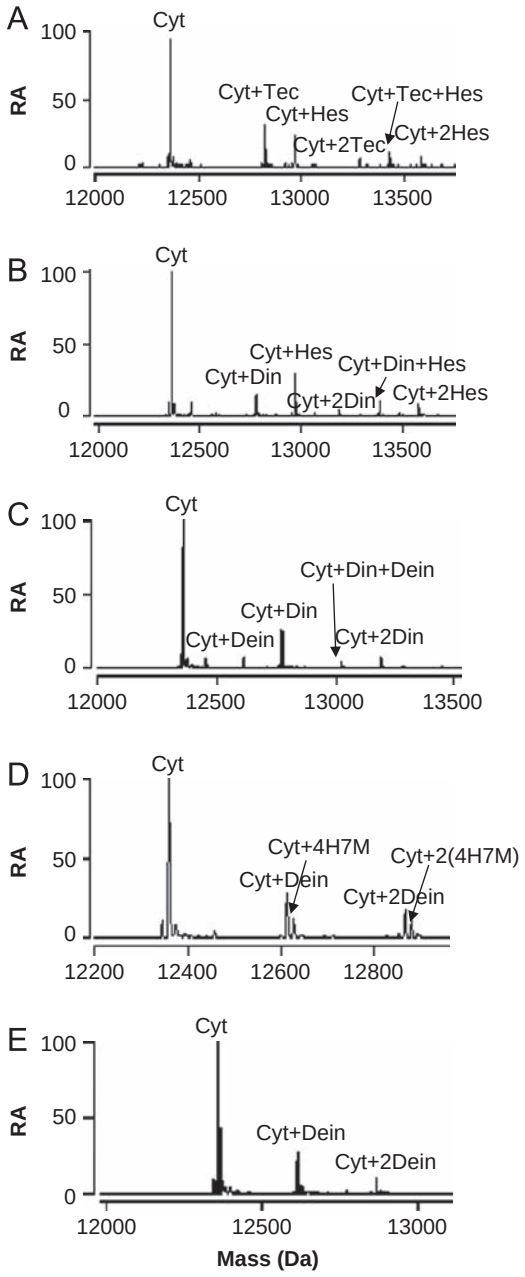

Fig. 4. Competition experiments of cytochrome-c binding to two ligands at a 1:5:5 ratio: (A) tectoridin+hesperidin (Tec+Hes); (B) hesperidin+daidzin (Hes+Din); (C) daidzin+daidzein (Din+Dein); (D) daidzein+7-methoxy-4′-hydroxyisoflavone (Dein +4H7M) and (E) daidzein+4′-methoxy-7-hydroxyisoflavone (Dein+4M7H).

lower *Kd* value represents higher binding affinity, the calculated *Kd* values are consistent with the order of binding affinities obtained in Section 3.3.

Based on the above results, Tec has the highest binding affinity while 4M7M has the lowest one. The three flavonoid glycoside ligands are more favoured to bind with Cyt-c than the four flavonoids, probably because their glycosyl units play important roles in the binding. The –OH groups of the glycosides could readily interact with amino acids in proteins via hydrogen bonding. With the absence of glycan, flavonoids such as Dein, 4H7M and 4M7H can still form noncovalent complexes with Cyt-c. Dein has two –OH groups, one at C4′ and the other at C7. Its binding affinity to Cyt-c is also higher than 4H7M and 4M7H which contain only one –OH group (the other –OH has been replaced by a –OCH_3 substitution). When both –OH groups are replaced by –OCH_3, the resulting molecule is 4M7M, which was not observed to form a stable complex with Cyt-c. It is interesting to note that 4H7M and 4M7H are a pair of isomers having the same molecular weight and size, and their only structural difference is that the –OH and –OCH_3 substituents at the C4′- and C7-positions are switched. However, the binding affinity of 4H7M with Cyt-c was found to be higher than that of 4M7H. This result may show that the –OH located at C4′ is a more active ligand than when it is at C7. Thus the SDB method with ESI-MS allows us to explore the effective binding of functional groups in flavonoid glycosides and flavonoids bound to Cyt-c. The binding modes and binding sites for the interactions of flavonoid glycosides/flavonoids and Cyt-c have been further investigated and confirmed by molecular docking simulations.

Table 1
Kd_1, Kd_2 and *RSD* results for the complexes of cytochrome-c and tectoridin (Tec)/hesperidin (Hes)/daidzin (Din)/daidzein (Dein)/7-methoxy-4′-hydroxyisoflavone (4H7M)/7-hydroxy-4′-methoxyisoflavone (4M7H).

Complexes	$Kd_1 \times 10^{-5}$ (mol/L)	*RSD* ($n=15$)	$Kd_2 \times 10^{-10}$ $(mol/L)^2$	*RSD* ($n=15$)
Cyt+Tec	1.208	0.044	0.901	0.24
Cyt+Hes	1.371	0.030	0.858	0.15
Cyt+Din	2.012	0.031	2.316	0.10
Cyt+Dein	2.554	0.025	2.939	0.050
Cyt +7M4H	4.740	0.038	n/a	n/a
Cyt +4M7H	7.331	0.029	n/a	n/a

3.5. Study of the interactions between flavonoid glycoside/flavonoid SDB ligands and Cyt-c by molecular docking

Computational methods are often used for the characterization and virtual screening of ligands binding to targeted proteins [44–47]. We here used an iGEMDOCK molecular docking method [47] in this study to supplement the ESI-MS experiments for gaining an insight into the interactions between Cyt-c and the flavonoid glycoside/flavonoid ligands. The protein–ligand interaction profile for each complex is visualized in Fig. 5 (the structures are given by the simplified ball–stick pictures). They display the interactions of the conserved interacting residues in protein and the functional groups of the ligand molecule. The flavonoid glycoside/flavonoid molecules form H-bonds with specific amino acid residues in Cyt-c. The preferred binding sites and the total energy of the formed H-bonds (calculated by MD) are given in Table 2.

The order of ligands arranged by the stabilities of H-bonds is Tec > Hes > Din > Dein > 4H7M > 4M7H, which is in good agreement with the order of binding affinities determined by ESI-MS analysis. This result may indicate that hydrogen bonding plays the major role in the formation of noncovalent complexes between Cyt-c and flavonoid glycoside/flavonoid ligands. Similar phenomena were observed in our previous study on noncovalent complexes of Cyt-c and saikosaponins [18] The docking results by iGEMDOCK show that Tec forms H-bonds with four amino acids of Cyt-c (i.e. S-Arg-38, M-Gly-41, S-Tyr-48 and S-Trp-59). The prefix "S-" indicates that the amino acid is located on the side chain and "M-" the main chain. The total energy of these four H-bonds in the Tec–Cyt-c complex is −28.70 kcal/mol. Hes also forms H-bonds with four amino acids (i.e. M-Cys-17, S-His-18, S-Trp-59, M-Lys-79), and the total energy of these H-bonds is −25.90 kcal/mol, higher than that of Tec–Cyt-c complex. From the binding profiles (Fig. 5B), distances between Hes and the two side chain amino acids, S-His-18 and S-Trp-59, are large (above 3.30 Å), and so the corresponding H-bonds formed are very weak. Hes has one more monosaccharide unit than Tec, and so it could form more H-bonds with

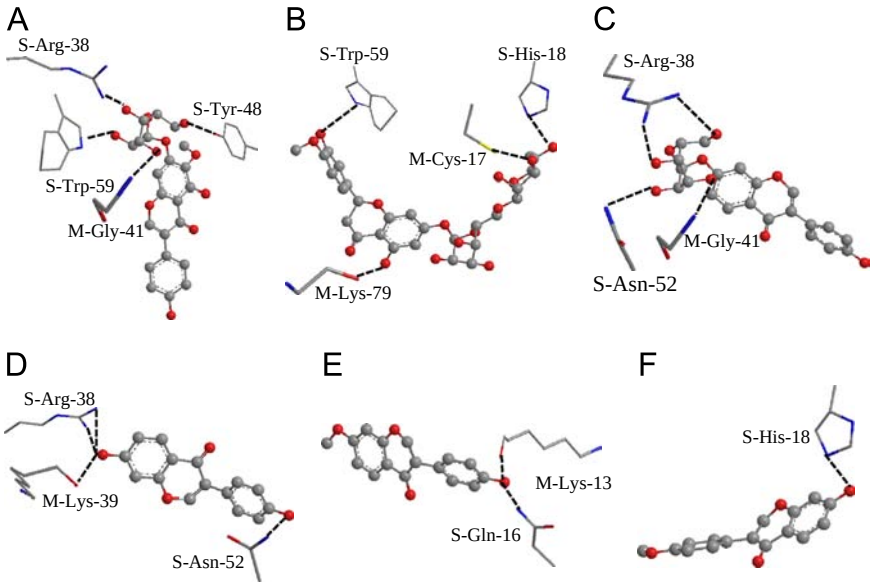

Fig. 5. Interaction profiles obtained by iGEMDOCK molecular docking: (A–F) Cyt-c–ligand interaction profiles, showing the H-bonds formed between the conserved interacting amino acid residues and each of the flavonoid glycoside/flavonoid ligands. (Oxygen atoms are marked in red and nitrogen atoms in blue.). (A) Tec + Cyt-c (B) Hes + Cyt-s (C) Din + Cyt-c (D) Dein + Cyt-c (E)4H7M + Cyt-c (F) 4M7H + Cyt-c.

Table 2
The total energy of hydrogen bonds and the interacting binding amino acid residues between cytochrome-c and tectoridin (Tec)/hesperidin (Hes)/daidzin (Din)/daidzein (Dein)/7-methoxy-4′-hydroxyisoflavone (4H7M)/7-hydroxy-4′-methoxyisoflavone (4M7H).

Ligand	Total H-bond energy (kcal/mol)	Potential binding sites
Tec	−28.70	S-Arg-38; M-Gly-41; S-Tyr-48; S-Trp-59
Hes	−25.90	M-Cys-17; S-His-18; S-Trp-59; M-Lys-79
Din	−20.40	S-Arg-38; M-Gly-41; S-Asn-52
Dein	−9.80	S-Arg-38; M-Lys-39; S-Asn-52
7M4H	−6.0	M-Lys-13; S-Gln-16
4M7H	−3.5	S-His-18

Cyt-c. However its larger size hinders an approach to the side chain amino acids and lowers the stability of the Cyt-c–Hes complex, as compared to Cyt-c–Tec. Din is able to form more H-bonds with the amino acids in Cyt-c than its flavonoid homologs, i.e. Dein, 4H7M and 7H4M, which contain no glycosyl moieties. Dein forms three H-bonds via the C4′-OH group with S-Arg-38 and M-Lys-39, and only a weak interaction with S-Asn-52 via the C7-OH. 4H7M forms two H-bonds with M-Lys-13 and S-Gln-16, respectively. 7H4M only interacts weakly with one amino acid, namely S-His-18. This may explain why the –OH group at C4′ forms more stable H-bonds with the amino acids than it does at C7, which is consistent with the ESI-MS observations.

Hence, MD results reveal that not only the presence of –OH groups in the ligand are important for H-bonding between Cyt-c and flavonoid glycosides/flavonoids, the structure size and position of the interacting functional groups in the ligand also affect the stability of H-bonds and so influence the binding affinity of noncovalent protein complexes. In addition, we have observed that the preferred binding sites of amino acids in Cyt-c are different for different ligands, depending on the amino acids available for interacting with accessible –OH groups. Flavonoid glycoside ligands have higher binding affinities than those of flavonoids, because their glycosyl components are capable to provide more –OH links than the latter, making for more facile interactions with proximate amino acids, via H-bonding.

4. Conclusions

The SDB–ESIMS–MD method developed in this paper permits an investigation of the interactions between protein and ligands, and allows for the determination of binding affinities, dissociation constants and the prediction of effective binding sites and binding modes. Using this method, the interaction of Cyt-c with flavonoid glycosides and flavonoid homologs were characterized and quantified in this work. The bindings for a series of structurally-different flavonoid–Cyt-c complexes were compared in detail. The order of relative binding affinities of the selected ligands with Cyt-c was measured by ESI-MS as Tec > Hes > Din > Dein > 4H7M > 4M7H > 4M7M. This is consistent with the order of total H-Bond energy given by molecular docking, indicating that Cyt-c interacts with these ligands primarily via H-bonds. MD simulations also provided the specific H-bond interaction sites of the amino acid residues in Cyt-c that bind to the functional groups of flavonoid-ligand. Therefore, interaction profiles of protein complexes obtained by MD simulations further complemented the ESI-MS experiments in providing more detailed binding information. Dissociation constants *Kd*s were determined quantitatively for the flavonoid–Cyt-c complexes and the trend of Kd_1 is in good agreement with the relative binding affinities obtained by the ESI-MS competition experiments. The consistency of results obtained from different experimental and computational measurements demonstrates that this method is a reliable and valid method for the study of protein–ligand interactions. The SDB–ESIMS–MD method reported here, combining systematic structure-differential bindings with mass spectrometry and computational docking, can be applied to the study of a broad range of noncovalent protein complexes.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.061.

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