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How to conduct and interpret ITC experiments accurately for cyclodextrin–guest interactions

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Isothermal titration calorimetry (ITC) is one of the most interesting methods for the characterization of the interaction mechanisms of cyclodextrins (CDs) with drugs. In this review we explain how to conduct ITC experiments correctly for CD–guest interactions, how to choose an accurate fitting model for the titration curve and how to interpret carefully the ITC results. Finally, the use of ITC for the characterization of CD-containing nanoparticles is discussed.

The isothermal titration calorimetry (ITC) technique is based on the measurement of the heat generated or absorbed upon the interaction between two molecules. Data derived from ITC were obtained in many areas from chemistry to cellular biology [1–3]. ITC was used for the characterization of polyelectrolyte complexes [4], adsorption on vesicles [5], self-associating systems [6], micellar-based systems (Fig. 1a) [7–9], nucleic acid interactions with multivalent cations (Fig. 1b) and the characterization of different functionalities carried by nanoparticles (Fig. 1c). A particular interest of ITC concerns its use for the characterization of CD–guest interactions (Fig. 1d) [10–15].

Shaped as a hollow truncated cone, CDs are cyclic oligosaccharides of D-(+) glycopyranose units all in chair conformation, linked by α-(1,4) glucosidic bonds. The outer side is formed by the secondary 2- and 3-hydroxyl groups and the narrow side by the primary 6-hydroxyl groups. Owing to this conformation, CDs have hydrophilic outer surfaces and lipophilic inner cavities, which enables CDs to interact with the less polar or hydrophobic part of the guests. As a result of molecular complexation phenomena, CDs are used to increase the solubility of poorly water-soluble molecules without co-solvents, surfactants and complex-forming agents [10–20]. Furthermore, recent studies have highlighted the use of CDs to modulate the amphiphilicity of block copolymers enabling the formation [21–23] or disruption [24] of micelles or the self-assembly of double hydrophobic [25] or hydrophilic block [26] polymers. Owing to the knowledge of the polymer–CD interactions, different kinds of self-assembled drug delivery systems were designed, ranging from nanoparticles [27] to hydrogels [28].

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Knowledge of the binding constants and the thermodynamic parameters of the interaction of a guest with CDs are of central importance for understanding the phenomena of molecular recognition. This can be achieved by using a wide variety of experimental methods, including nuclear magnetic resonance [29], fluorescence, chromatography, electrophoresis, phase solubilization studies, potentiometry and circular dichroism spectroscopy. However, each technique has its drawbacks in comparison with ITC. For example, ITC led to the characterization of low affinity constants (lower than $10\ M^{-1}$) [30] and high affinity constants (higher than $10^7\ M^{-1}$) [31] making it a preferred method in comparison with other analytical methods.

ITC is the most sensitive method available for the determination of the stoichiometry of the interaction (N), the affinity constant (K) (also called stability or association constant) and the enthalpy change (ΔH), which reflects the heat released or taken up during the interaction. Furthermore, the entropy (ΔS) and the Gibbs free energy of the process (ΔG) can be calculated from the ITC data [3,10–16,32]. ITC measurement has become an alternative method for directly determining the thermodynamic parameters previously calculated from the van't Hoff equation [1].

However, an adequate model has to be applied to fit the titration curve and the results extracted from the ITC experiments should be interpreted carefully. In this review we focus on literature works related to the use of ITC for the characterization of CD–guest interactions, we try to help the reader to analyse the thermodynamic parameters of these interactions and we give some relevant examples about how to understand the mechanism of molecular interaction between the guests and the CDs by combining ITC experiments with other physicochemical characterization methods.

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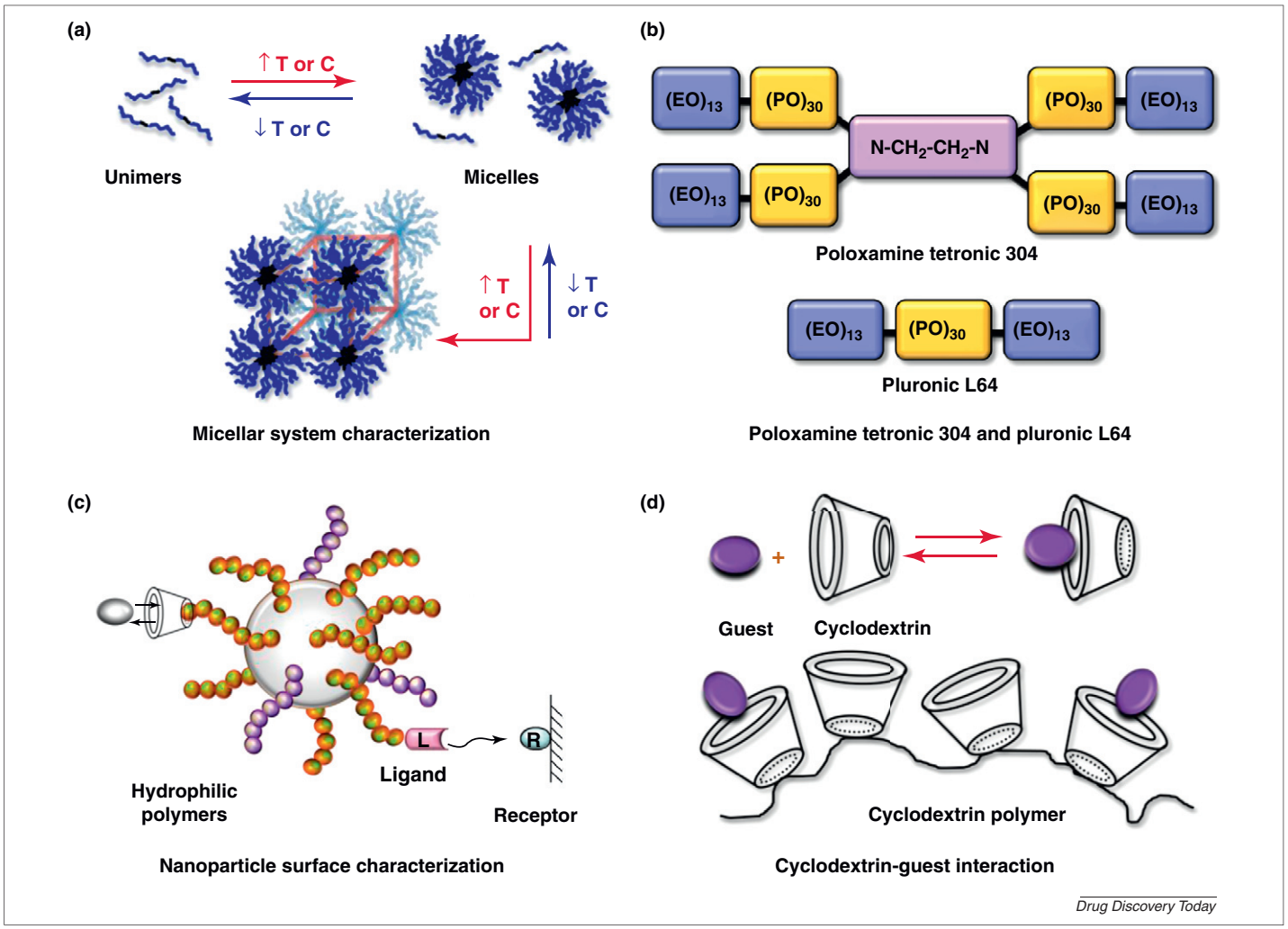

FIGURE 1

Drug delivery systems that can be characterized by using isothermal titration calorimetry. **(a)** Micellar system characterization. Pluronic F127 micelles were formed by increasing the temperature or the concentration of the pluronic [7–9]. **(b)** Characterization of the interaction of surfactants with nucleic acids [9]. **(c)** Characterization of the surface of nanoparticles. **(d)** Cyclodextrin–guest interactions [10–15]. *Abbreviations*: C: concentration of pluronic F127; T: Temperature of the experiment.

A typical ITC experiment

From a technical point of view, ITC experiments are relatively simple. They consist of consecutive injections of small volumes (5–10 μL) of an aqueous solution of CD (contained in the stirring syringe) and guest molecule solution which is contained in the measurement cell (Fig. 2). More technical details on the description of ITC apparatus and the mode of operation were previously described [1]. The measurement cell is maintained at a constant temperature and the heat of the process occurring during dilution is monitored for each injection and plotted as a function of time. In commercial ITC apparatuses, the experimentally accessible range of temperature is comprised between 2 and 80°C, but experimental temperatures investigated are usually 4°C [10,12], 25°C [11] or 37°C [10,12].

The CD and the guest concentrations in the syringe or in the measurement cell could be adjusted depending upon several factors, such as the solubility and the affinity of the CD–guest interaction. The concentrations of the guest and the CD have to be adjusted to obtain titration curves that can be fitted properly. The observed heat signals obtained upon the titration are dependent on the concentrations used.

Generally, the ITC experiment is conducted in aqueous media, such as water or phosphate buffer offering the possibility to investigate the effect of pH changes on the interaction [16,33–35]. However, because most of the studied guest molecules have limited water solubility, a major limitation is related to the poor concentration of the guest in aqueous media, making it difficult to extract accurate information from ITC experiments. In such situations, a co-solvent can be used to increase the guest concentration in the measurement cell. For example, a water/ethanol (50/50%, v/v) mixture was used to investigate the interaction between docetaxel (Dtx) and methyl-β-CD (Me-β-CD) [15] and paclitaxel with bis-β-CDs [36].

Different binding models used to fit the CD–guest interaction curve

When CD is added to the guest solution, the two molecules interact and the heat is proportional to the amount of CD added to the guest solution. As the population of the guest molecule in

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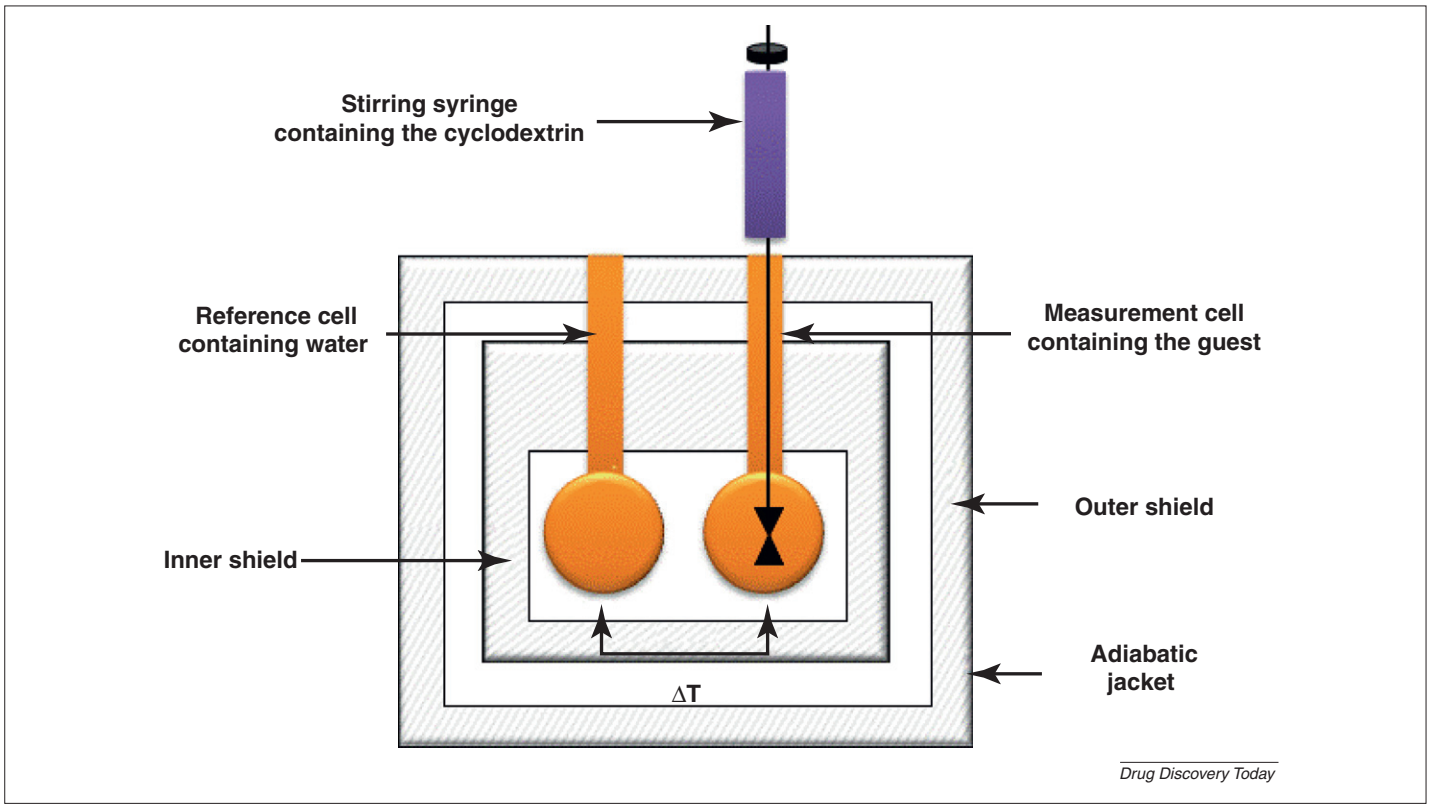

FIGURE 2
The isothermal titration calorimetry apparatus.

the cell becomes more saturated with CDs, the heat signal diminishes progressively (Fig. 3a). After integrating the heat as a function of the molar ratio between the two reactants, the titration curve should be corrected for the dilution and for possible protonation effects. The integration curve (Fig. 3b) can then be fitted to various models making it possible to determine the association constants, the stoichiometry, and the thermodynamic profiles of the interaction.

In many research works, the titration curve was fitted to the one-set of sites model [37,38]. The simplified 1:1 complexation interaction of the guest with a CD host in aqueous media can be written as follows:(1)$G + CD \overset{K}{\rightleftarrows} G \cdot CD$The binding constant for a 1:1 complexation of the CD with the guest molecule is expressed by Eqn (2):

$$K = \frac{[G \cdot CD]}{[G][CD]} \quad (2)$$

where [CD], [G] and [G·CD] are the concentrations of the CD, the guest molecule and the inclusion complex, respectively. At equilibrium, the change in Gibbs free energy for the formation of [G·CD] inclusion complex is calculated according to Eqn (3) [1]:

$$\Delta G = -RT \ln K \quad (3)$$

where R is the gas constant ($8.314\ J\ K^{-1}\ mol^{-1}$) and T is the absolute temperature at which the interaction takes place, expressed in degrees Kelvin.

However, when the fit of the integration curve to a simple 1:1 model is unsuccessful, other stoichiometries could be found [38–44]. Unsuccessful fit of the integration curve is reflected by poor chi square values and/or inappropriate values for K and/or thermodynamic parameters. For example, 1:2 stoichiometry was found for the interaction of paeonol with β-CD [45]. A stoichiometry of 2:1 was found for the interaction of cationic gemini surfactants with α-CD [45], whereas the interaction of chlorhexidine with β-CD occurred according to a stoichiometry of 1:4 [38].

In the one-set of sites model, all the binding sites are considered to be identical as no distinction between the interacting sites is made. In this context, it was also possible to fit the titration curve to the two-set of sites model in which the association constants are expressed by the following equations:

$$K_1 = \frac{[G \cdot CD_1]}{[G][CD_1]} \qquad K_2 = \frac{[G \cdot CD_2]}{[G][CD_2]} \quad (4)$$

However, a major source of systematic errors in the interpretation of ITC data is related to the inadequate application of the two-sets of sites model, which assumes that the two interactions occur independently one from another. In some complex binding situations, such as the existence of binding co-operativity, the curves cannot be fitted to a particular model. In these cases, a general model-free methodology should be applied [46]. For example, we applied a 'sequential model' in the ITC software to fit the titration curve of Me-β-CD with Dtx [15]. The *tert*-butyl and C_{30}–C_{35} phenyl groups interacted with one Me-β-CD and the C_{24}–C_{29} phenyl group interacted with a second Me-β-CD (Fig. 4). The combination of nuclear magnetic resonance analysis and molecular modeling to ITC results enabled us to demonstrate that the first Me-β-CD_1 molecule which binds to Dtx, always binds to site 1 (both *tert*-butyl and C_{30}–C_{35} aromatic group; Eqn (5)), then the second Me-β-CD_2 molecule which binds to Dtx, always binds to site 2 (C_{24}–C_{29}

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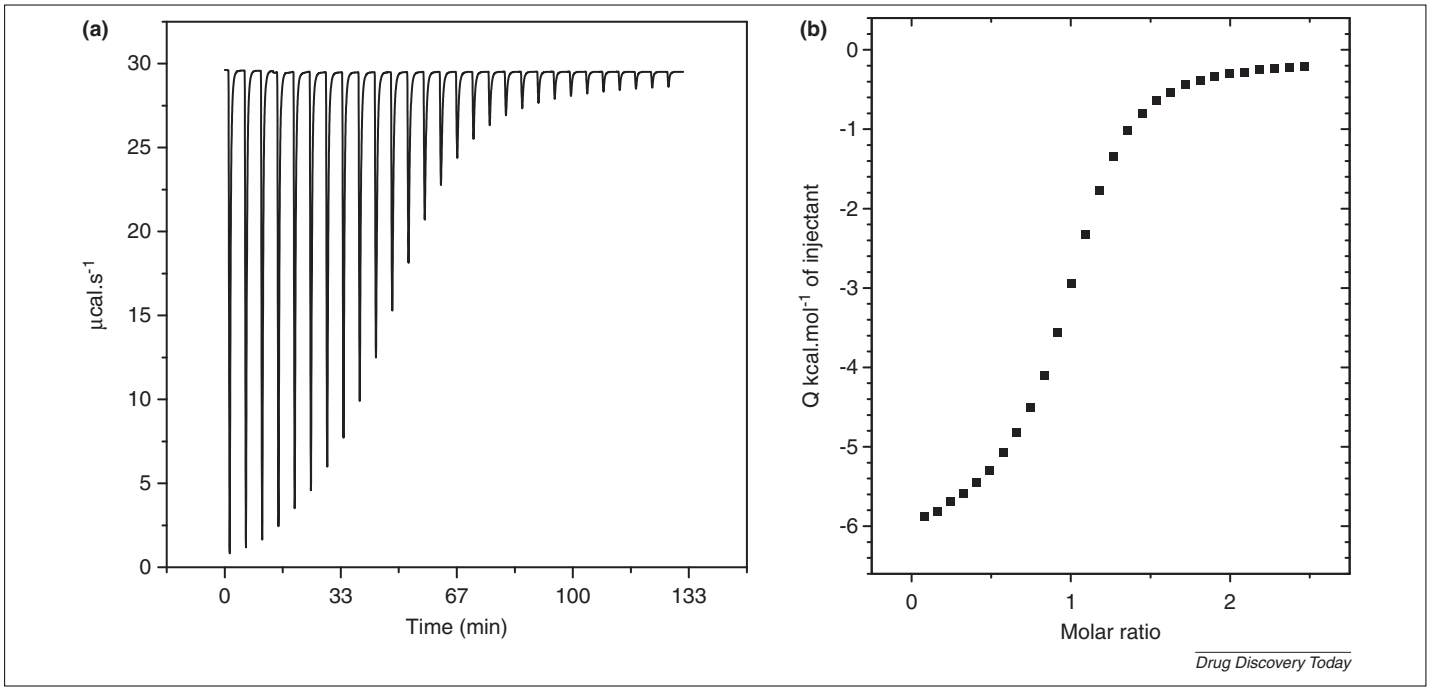

FIGURE 3

Typical isothermal titration calorimetry data obtained for the binding interaction in water at 25°C of 1-adamantylamine (1.3 mM) to β-CD (15 mM). **(a)** Shows exothermic heat releases upon injection of 10 μL aliquots of β-CD into 1-adamantylamine solution. **(b)** Shows integrated heat data, giving a differential binding curve.

aromatic group; Eqn (6)) association constants were expressed by Eqn (7).

The ΔG calculated from Eqn (3) is more negative for the first interaction than the second interaction (ΔG_1: -16.38 kJ mol^{-1} and ΔG_2: -13.15 kJ mol^{-1}) because generally, stronger interactions (reflected by higher values of K) are also more spontaneous (reflected by negative ΔG values) than the weaker interactions [15,47]. Sequential binding model was also applied for the complexation of guests with native CDs [48,49], CD dimers and CD polymers [50–52].

$$\text{Dtx} + \text{Me-β-CD}_1 \overset{K_1'}{\rightleftarrows} \text{Dtx} \cdot \text{Me-β-CD}_1 \quad (5)$$

$$\text{Dtx} \cdot \text{Me-β-CD}_1 + \text{Me-β-CD}_2 \overset{K_2'}{\rightleftarrows} \text{Me-β-CD}_2 \cdot \text{Dtx} \cdot \text{Me-β-CD}_1 \quad (6)$$

$$K_1' = \frac{[\text{Dtx} \cdot \text{Me-β-CD}_1]}{[\text{Dtx}][\text{Me-β-CD}_1]} \qquad K_2' = \frac{[\text{Me-β-CD}_1 \cdot \text{Dtx} \cdot \text{Me-β-CD}_2]}{[\text{Dtx} \cdot \text{Me-β-CD}_1][\text{Me-β-CD}_2]} \quad (7)$$

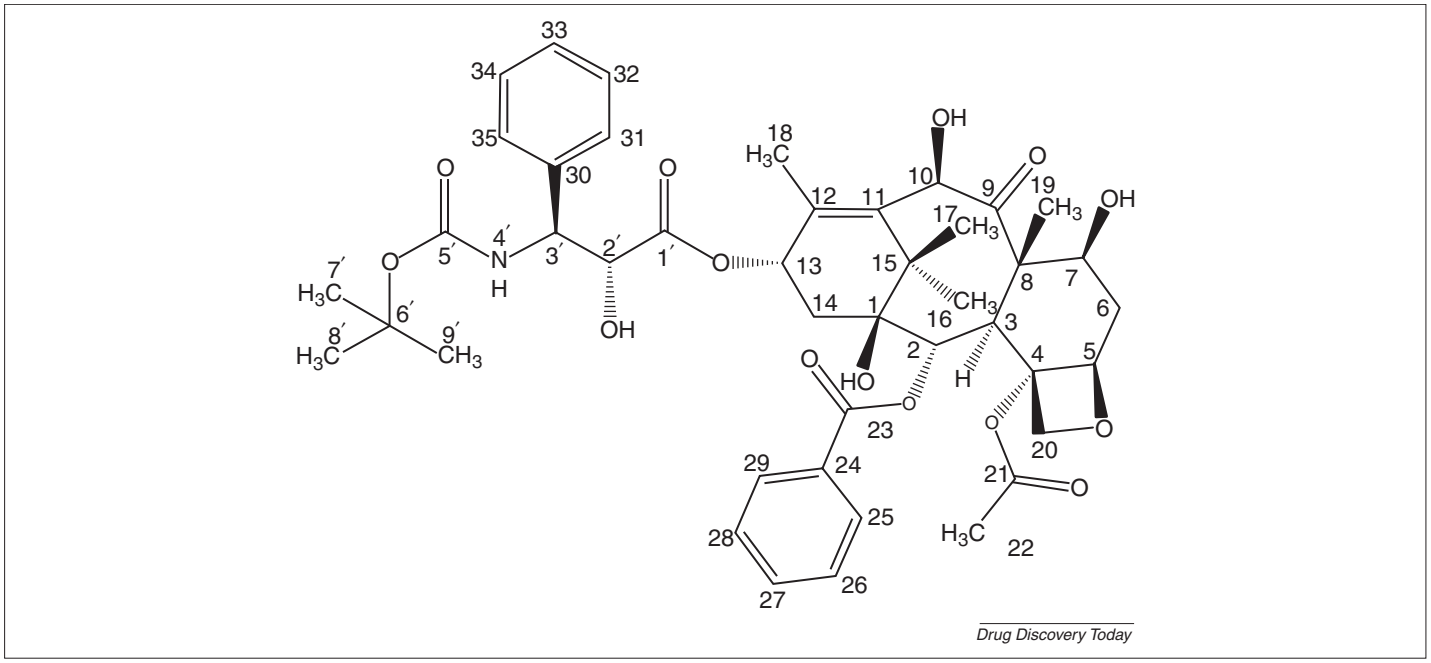

FIGURE 4

Chemical structure of docetaxel.

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Thermodynamics of CD–guest interactions

Manifold mechanisms have been proposed as the driving force for the formation of the CD inclusion complex with guest molecules. The thermodynamic changes observed were related to the effect of water. Indeed, it is suggested that the slightly apolar CD cavity is occupied by water molecules. In the presence of hydrophobic molecule which can interact with the CD cavity, water can be substituted by the hydrophobic part of the guest molecule which is less polar than water resulting in a more stable lower energy state (Eqn (8)).

The inclusion complexation of guest molecules by CDs in aqueous solutions results in a substantial rearrangement and removal of the water molecules originally solvated to both the CD and the guest molecules, and this process also induces the release of water molecules from the CD cavity into the bulk water. Taking into account the initial water molecules included into the CD, the 1:1 complexation interaction of the guest with a CD host in aqueous media can be written as follows:

$$G \cdot gH_2O + CD \cdot hH_2O \overset{K}{\rightleftarrows} G \cdot CD \cdot (g + h - i)H_2O + iH_2O \quad (8)$$

where g represents the number of water molecules interacting with the free guest, h the number of tightly bound hydration water molecules inside the free CD cavity, and i the net displacement of water upon complexation [53,54]. Although the estimation of the number of water molecules displaced upon binding is difficult to be obtained unambiguously, a recent work led to estimate the i in the range of 15–25 water molecules released upon adamantane binding to β-CD [55].

The thermodynamic quantities obtained for the inclusion complexation by CDs are a consequence of the contributions of these interactions. The ΔH obtained from ITC analysis represents a global heat resulting from the interaction of the guest with the CD. Large positive ΔS changes usually arise from the significantly important translational and conformational freedoms of host and guest upon complexation [55,56]. The entropy of the interaction can be calculated according to Eqn (9) from the K and the ΔH derived from the fit of the ITC titration curve:

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (9)$$

The principal factors involved in binding are van der Waals and hydrophobic interactions. The study of ΔH and ΔS leads to the differentiation between the different types of non-covalent forces involved in the guest–CD interactions. van der Waals interactions are enthalpy-driven processes with minor favorable or unfavorable entropies of interaction ($|\Delta H| > |T\Delta S|$) [57]. Hydrophobic interactions are entropy-driven, where the entropy of the interaction is large and positive whereas the enthalpy of the process is small ($|\Delta H| < |T\Delta S|$) [58].

Other intermolecular interactions, such as hydrogen bonding and electrostatic interactions could contribute to the inclusion complexation behaviours of CDs to guest molecules [59]. It was reported that the interaction of positively charged CDs with neutral or charged guests revealed the presence of electrostatic, van der Waals, hydrogen bonding and hydrophobic interactions [30,34].

In agreement with the vant' hoff equation (Eqn (10)), ITC experiments demonstrated that the temperature had a significant effect on CD–guest association constant and thermodynamics.

$$\mathrm{Ln}\, K_{vH} = -\frac{\Delta H_{vH}}{RT} + \frac{\Delta S_{vH}}{R} \quad (10)$$

Let us examine for example the effect of temperature on the association constant of the interaction of benzophenone with β-CD. Much higher affinities and much stronger interactions were obtained when the temperature of the experiment was decreased from 37 to 4°C [10]. For temperatures 25 and 37°C, the association processes were predominantly enthalpy driven ($|\Delta H| > |T\Delta S|$) and the association constants were 2680 and 1560 M^{-1}, respectively [10]. However, when the temperature of the experiment was low (4°C), the interaction of benzophenone with β-CD was entropy driven ($|\Delta H| < |T\Delta S|$) and the association constant was high (3770 M^{-1}). The entropic gain usually arises from the important translational and conformational freedoms upon the interaction of the guest with CD cavity. Furthermore, the desolvation upon benzophenone inclusion and the induced dehydration from peripheral hydroxyl groups of CD cavity when the temperature of the experiment is lowered appear to be responsible for the entropic gain [33,34].

Generally, little variation of the ΔG was reported when temperature was changed [10,16]. This can be attributed to the compensation of the enthalpic changes by the $T\Delta S$ variations. For example, a linear relationship was obtained when plotting ΔH against $T\Delta S$ for the different reactions of complexation of benzophenone with β-CD and poly-β-CD [10] or for the complexation of (+)-usnic acid with γ-CD [16] confirming the existence of an enthalpy–entropy compensation effect. Enthalpy–entropy compensation owing to temperature change can be shown to be a consequence of thermal heat capacity (ΔC_p) effects, which are related to water reorganization upon transfer of the guest from bulk water to CD cavity [56]. The ΔC_p was calculated from the slope of the curve $\Delta H = f(T)$ according to Eqn (11).

$$\Delta C_p = \frac{\Delta H_{T2} - \Delta H_{T1}}{T_2 - T_1} \quad (11)$$

Enthalpy–entropy compensation was also reported when water was progressively replaced by a solvent [59,60]. These differences in thermodynamic parameters could result from the interactions between the solvent and the CD. For example, the presence of hydrogen bonds between the guest molecule and the solvent results in more rigid structure of the inclusion complex and, in turn, decreases the entropy of the interaction [61,62]. The increase of the enthalpy of the interaction results from small variations of the ΔG [57].

ITC for the characterization of nanoparticles bearing CDs

Before their administration *in vivo*, nanoparticle formulations should combine several different properties, such as efficient drug loading, controlled drug release and targeting. ITC was used for the characterization of nanoparticle surface bearing for example CDs [63,64], ligands able to recognize specific molecular structures at the target site [65]. However, the intravenous administration of nanoparticles is involved in their recognition by plasmatic proteins (opsonins) followed by their recognition by the macrophages. Hydrophilic moieties, such as polysaccharides or poly(ethylene glycols) were able to repulse the opsonins and avoid adsorption. ITC was used to estimate the interaction of the nanoparticles with bovine serum albumin as model protein [66–68].

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Particularly, the incorporation of CDs into nanoparticles should increase low drug loading and enable the incorporation of ligand-terminated molecules owing to the interaction between drugs and CDs. For example, ITC was used with the aim to confirm the presence of β-CDs at the surface of gold nanoparticles by their interaction with adamantyl-terminated guest molecules [63].

In a work conducted by Martinez-Barobosa *et al.* [64] the synthesis and the characterization of a novel poly(γ-benzyl L-glutamate)-β-CD polymer was carried out. ITC experiments were used to show unambiguously that the stoichiometry of the interaction was 1:1 and that 20% of the CDs remained functional within the nanoparticles [64].

Concluding remarks

ITC is a potentially valuable technology for characterizing CD–drug interactions. It provides an interesting quantitative method for the characterization of CD–drug interactions in a label-free manner. However, an adequate fitting model should be used, and the data derived from ITC experiments should be interpreted carefully considering the temperature, the medium composition and other parameters not related to the interaction.

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