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New challenges for pharmaceutical formulations and drug delivery systems characterization using isothermal titration calorimetry

Kawthar Bouchemal

Université Paris-Sud 11, School of Pharmacy, UMR CNRS 8612, 5 Rue J.B. Clément, 92296 Châtenay Malabry, France

Long viewed as the 'method of choice' for characterizing thermodynamics and stoichiometry of molecular interactions, with high sensitivity, isothermal titration calorimetry (ITC) has been applied to many areas of pharmaceutical analysis. This review highlights ITC employment to measure binding thermodynamics and their use for pharmaceutical formulations and drug delivery system characterization particularly cyclodextrin–guest interactions, investigation of micellar-based systems, polyelectrolytes, nucleic acid interactions with multivalent cations and the optimization of DNA targeting and delivery. Furthermore, the potential of ITC for the characterization of different functionalities carried by nanoparticles as well as their interaction with living systems was outlined.

Introduction

Nowadays, isothermal titration calorimetry (ITC) is becoming the method of choice for characterizing intermolecular interactions and recognizing reactions with exquisite sensitivity, since both low- and high-affinity interactions can be quickly and accurately characterized using ITC. In this technique, reactive materials can be studied not only in solution, but also in the form of particulate suspensions, because the turbidity or the color of the samples has no influence on the measurement. Furthermore, the thermodynamic parameters of the interaction can be measured without immobilization, modification or labeling of the binding partners and without molecular weight restrictions. As far as the general principle is concerned, the ITC technique is based on measurement of the heat that is generated or absorbed in an interaction between two molecules. ITC has been applied to determine the stability constants, stoichiometry, interaction enthalpies, and, under some conditions, entropies, Gibbs free energies and heat capacity changes.

Data derived from ITC have been obtained in many areas such as polymer chemistry [1–3], nanotechnology, cellular biology and biochemistry. Several papers and books have been published describing the applications of this technique [4,5] for the study of biological and bio-molecular recognition interactions [6,7], ligand binding [8], enzyme activity [9,10], biotechnology [11], drug discovery, design and development [12], protein–protein [13–15], protein–receptor [16], lipid–lipid [17] and lipid–small molecules interactions [18].

The analysis of thermodynamic parameters of interactions provided the knowledge of manufacturing conditions, which are essential to exert control over the design and subsequent performance of pharmaceutical formulations. The analysis of thermodynamic parameters, combined with the study of physicochemical properties of the phenomena, allows an understanding of *how* the two molecules interact, and why they do so. How does thermodynamics drive binding and specificity? What thermodynamic processes control the conformational rearrangements of molecules? Which factors control the molecular interactions, and determine whether or not they take place?

Cyclodextrin–guest interactions

Cyclodextrins are cyclic oligosaccharides of D-(+) glycopyranose units obtained from the degradation of starch by enzymes produced by *Bacillus macerans*. The D-(+) glucopyranose units, all in chair conformation, are linked by α-(1,4) glucosidic bonds conferring to cyclodextrins the shape of a truncated cone, with the outer side formed by the secondary 2- and 3-hydroxyl groups and the narrow side by the primary 6-hydroxyl. Thanks to this conformation, cyclodextrins have lipophilic inner cavities and hydrophilic outer surfaces. So far, the most probable mode of binding involves the interaction of the less polar part of the guest molecule with the cyclodextrin cavity, while the more polar – and often

E-mail address: Kawthar.bouchemal@u-psud.fr.

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charged – group of the guest is exposed to the bulk solvent outside the wider opening of the cavity [19]. As a result of molecular complexation phenomena, cyclodextrins are used to increase the solubility of poorly water-soluble molecules without cosolvents, surfactants and complex-forming agents.

Knowledge of the binding constants and the thermodynamic parameters of the interaction are of central importance for understanding the phenomena of molecular interaction of a guest with cyclodextrins. Nowadays, a good deal of effort has been devoted to understand the thermodynamics of the complex formation between cyclodextrins and guests. This can be achieved by varying different environmental conditions and by using a wide variety of experimental methods including several types of spectroscopic techniques, ^{1}H NMR, fluorescence, solubilization studies and calorimetry. Of the various techniques mentioned above, high sensitivity titration calorimetry is the most modern and sensitive method available at the present time for the determination of thermodynamics of the host–guest interaction. ITC shows whether an association process occurs and allows the evaluation of the association constant (K), the enthalpy (ΔH) and the entropy (ΔS) of the interaction from which the Gibbs free energy of the process (ΔG) can be derived [20–25].

In a typical ITC experiment, aliquots of a concentrated solution of cyclodextrins are added in a time-controlled manner to a cell containing the interacting molecule, which is maintained at constant temperature. During cyclodextrin addition, the two materials interact and the observed released heat is directly proportional to the amount of binding compound added with the solution aliquot. As the population of interacting molecules in the cell becomes more saturated with cyclodextrins, the heat signal diminishes until only the background heat of dilution is observed (Fig. 1a). After integrating the heat as a function of molar ratio between the two reactants, it is possible to fit the integration curve to various models making possible to determine the association constants, the stoichiometry and a thermodynamic profile of the interaction.

Taking into account the initially included or interacting water molecules, the 1:1 complexation interaction of the guest with a cyclodextrin host may be written as follows:

$$\mathrm{G}{\cdot}g\mathrm{H_2O} + \mathrm{CD}{\cdot}h\mathrm{H_2O} \leftrightarrow \mathrm{G}\cdot\mathrm{CD}\cdot(g+h-i)\mathrm{H_2O} + i\mathrm{H_2O} \quad (1)$$

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 cyclodextrin cavity and i represents the net displacement of water upon complexation [19,26]. The binding constant for a 1:1 complexation of the conjugated cyclodextrin with the guest molecule is expressed by Eqn 2:

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

where [CD], [G] and [G·CD] are the concentrations of the cyclodextrin, the guest molecule and the inclusion complex, respectively. The change in Gibbs free energy, under arbitrary conditions, for the formation of A·B is expressed by the relationship $\Delta G = -RT\ln K$, where R is the gas constant (8.314 J K^{-1} mol^{-1}) and T is the absolute temperature of the interaction in Kelvin.

In many research works, thermodynamics of the complex formation with 1:1 host–guest stoichiometry was described

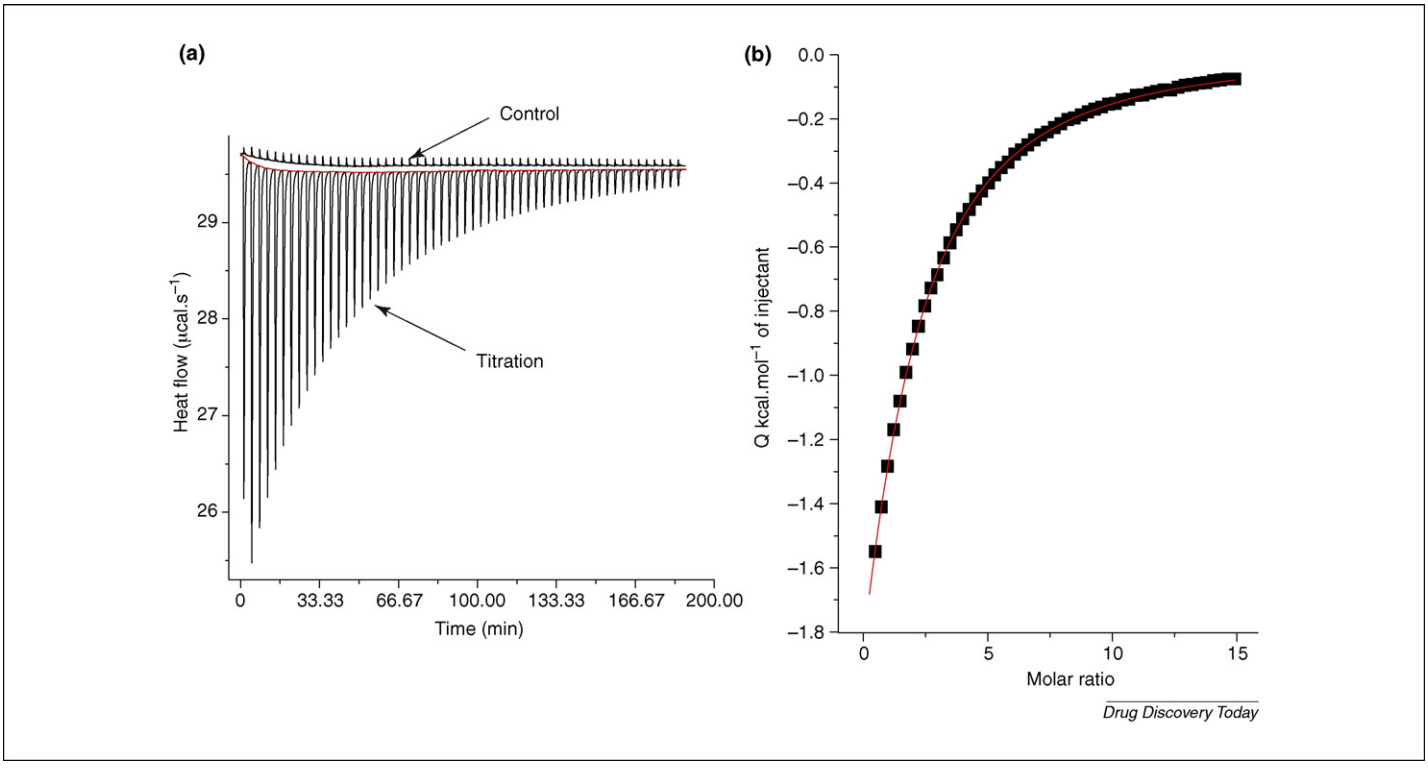


FIGURE 1

Typical ITC data obtained from the binding interaction of a guest to a cyclodextrin. The left-hand panel shows exothermic heat released upon injection of 5 μl aliquots of the cyclodextrin into the guest solution. The right-hand panel shows integrated heat data, giving a differential binding curve which was fitted to a standard single-site binding model yielding the following parameters N, K and ΔH.

[25,27,28]. In some cases, an unsatisfactory description or unsuccessful fit of the experimental data to a simple 1:1 host–guest complex model has led to the application of other models with more complicated stoichiometries such as, for example, a stoichiometry of 1:2 for the interaction of paeonol with β-cyclodextrin [22], and 2:1–6:1 for the interaction of cationic gemini surfactants with α-cyclodextrin [23]. Furthermore, complicated stoichiometries were obtained using supramolecular cyclodextrins based systems as previously described in many research works [24,29–31].

Whatever the stoichiometry obtained, the most described mechanism for the cyclodextrins–guest interaction is that, in an aqueous solution, the slightly apolar cyclodextrin cavity is occupied by water molecules which are energetically unfavored (polar–apolar interaction), and therefore can be readily substituted by appropriate 'guest molecules' which are less polar than water resulting in a more stable lower energy state. From a thermodynamic point of view, a stronger interaction of a molecule with the cyclodextrins is evidently expressed not only by a higher value of the binding constant, but also by a more negative value of ΔH and ΔG indicating that the interaction is stronger and more spontaneous, respectively.

Up to now, several driving forces have been proposed for the interaction of guests with cyclodextrins. van der Waals force and hydrophobic interactions related to the size/shape matching between guest molecule and cyclodextrin cavity are those among the several possible weak noncovalent interactions which provide the most crucial contributions toward the complexation of organic guests with cyclodextrins. The study of the enthalpy and the entropy leads to the differentiation between these two types of forces. Traditionally, hydrophobic interactions between two apolar molecules at room temperature have been known as entropy-driven processes, where the entropy of interaction is large and positive while the enthalpy of the process is small ($|\Delta H| < |T\,\Delta S|$) [32]. However, van der Waals interactions are usually enthalpy-driven processes with minor favorable or unfavorable entropies of interaction ($|\Delta H| > |T\,\Delta S|$) [19,33,34].

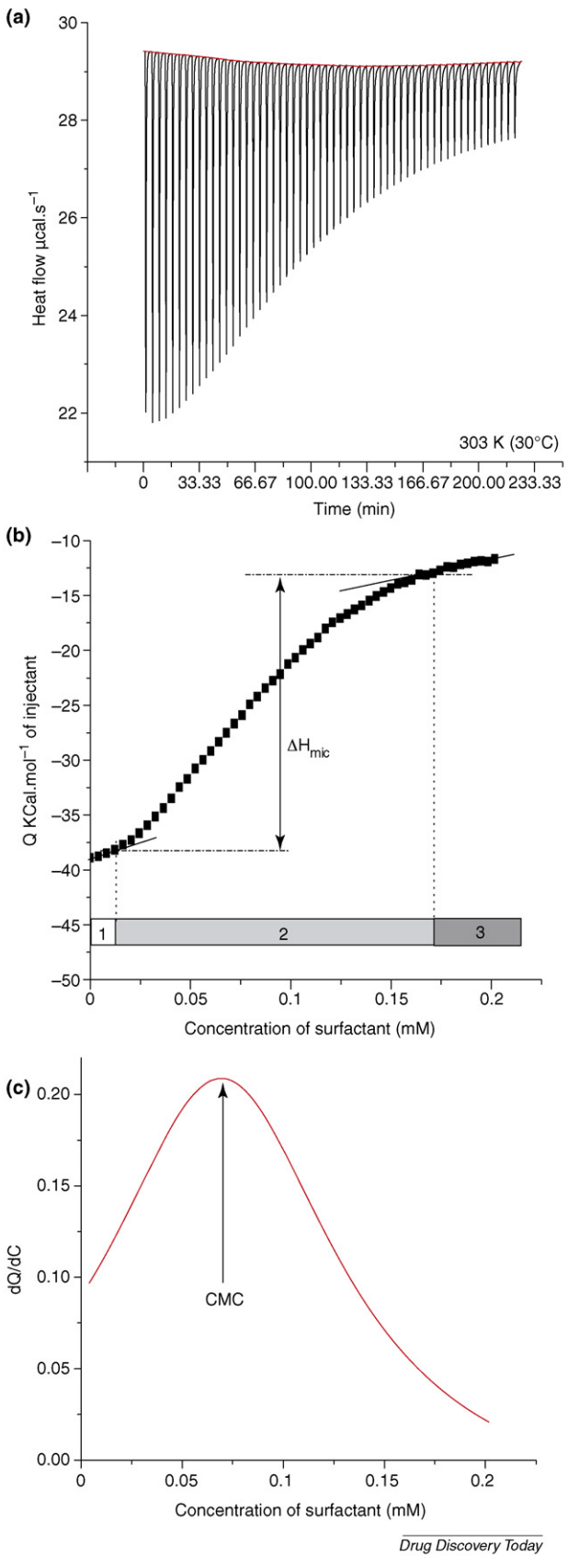
Besides hydrophobic and van der Waals forces, in some cases, other intermolecular interactions such as hydrogen bonding and electrostatic interactions contribute to the inclusion complexation behaviors of cyclodextrins, to varying molecules [35]. As a result, the electrostatic interactions between the hosts and the guests have attracted the interest of chemists during the design of functionally modified cyclodextrins [36]. It was reported that the interaction of aminated β-cyclodextrins possessing a positive charge with neutral or charged guests revealed the counterbalance between electrostatic and conventional intracavity interactions including van der Waals, hydrogen bonding and hydrophobic interactions [37–40]. It was also reported that the cationic aminated β-cyclodextrins could significantly alter the molecular-binding ability and selectivity of the parent β-cyclodextrins toward anionic molecules through cooperative electrostatic interactions, van der Waals and hydrophobic interactions between hosts and guests [41].

Investigation of micellar systems

It is well known that amphiphile molecules can aggregate in aqueous solution depending on the molecular structure of the surfactant, the concentration and the temperature. The critical micellization temperature (CMT) of various amphiphiles is a widely studied phenomenon and its determination has been achieved using different techniques such as dye solubilization method or differential scanning calorimetry experiments in which the enthalpy change related to the micellization process can be calculated from integration of the heat capacity versus the temperature [42]. For our knowledge, ITC is the only technique capable of measuring the critical micellization concentration (CMC) and the enthalpy of the micellization (ΔH_{mic}) (of course, equal in magnitude but opposite in sign to the heat of demicellization (ΔH_{demic})) of a surfactant in a single experiment without the necessity of any probe. In the literature, ITC was used as unique technique for the characterization of micellization behaviors [43,44] or used with other complementary techniques such as fluorescence spectroscopy [45], conductivity [46], potentiometric titration [45] and differential scanning calorimetry [47].

Typically, in an ITC experiment aiming to study the micellization process, a syringe containing micellar solution was progressively diluted in water contained in the sample cell. The concentration of micellar solution in the syringe was chosen in such a way that, with increasing surfactant concentration in the sample cell, the CMC was reached during the experiment. Generally, if a sigmoidal enthalpogram is obtained, it can be divided into three concentration ranges reflecting the following events (Figs 2 and 3): (i) after the first few injections, the final concentration of the surfactant molecules in the sample cell remains below their CMC and the weak enthalpic effects appear on account of the dissociation of the micelles to unimers and to the dilution of the resultant unimers. (ii) If more micellar solution of the surfactant is added to the sample cell, a clear increase in the heat is observed indicating that the added micelles are not dissociated; the critical micellar concentration is progressively approached at each injection (Fig. 3). The surfactant concentration in the sample cell is now above the CMC. (iii) If more micellar solution is added beyond the micellization concentration, the micelles no longer dissociate and the micellar solution is being diluted, leading to an observed heat output due to the micelles dilution, this is the third concentration range (Figs 2 and 3). From the calorimetric titration curve, and if a clear break of the curve is obtained, the ΔH_{mic} is calculated from the enthalpy difference of the two levels of the titration curve (Fig. 2b) and the CMC can be directly determined from the maximum value of the first derivative of the titration curve (Fig. 2c). Most amphiphiles like amino-acid-type surfactants [48], β-casein [49], sodium dodecyl sulfate [50–52], oligo(ethylene oxide) alkyl ethers [53,54] and bile salts, which are used, for example, as drug delivery systems [55–57] show only one extremum in the first derivative of the titration curve of the micellization experiment, which corresponds to one CMC value. However, other amphiphiles clearly show the appearance of two extrema, indicating two CMC values [58].

The experimental determination of the ΔH_{mic} and the CMC values allowed the calculation of other thermodynamics parameters. The standard Gibbs free energy change for the transfer of one amphiphilic molecule from solution to the micellar phase, also called the free energy of micellization (ΔG_{mic}), in the absence of electrostatic interactions is given by the expression $\Delta G_{mic} = RT \ln \mathrm{CMC}'$, where R and T have their habitual significations and CMC′ is the critical micellization concentration expressed in its



molar units [44,49]. The change in entropy associated with the micellization (ΔS_{mic}) can be calculated from the second law of thermodynamics [50,59] by using the Gibbs–Helmholtz equation $T\,\Delta S_{mic} = \Delta H_{mic} - \Delta G_{mic}$. Finally, the thermal heat capacity of micellization $\Delta C_{p,m}$ could be evaluated, on the basis of the following relationship:

$$\Delta C_{p,m} = \left(\frac{\partial \Delta H_m}{\partial T}\right)_p$$

Besides the determination of the CMC and the thermodynamics parameters of the micellization, ITC allowed the investigation of the interactions of surfactants with molecules such as drugs, polymers and proteins. Hereafter, we highlight some relevant examples about these interactions in relation with pharmaceutical formulation and drug delivery.

Drug–surfactant interactions

Despite the fact that surfactants are commonly used to increase the solubility of poorly water-soluble drugs, the interactions between drug and surfactant can be complex, making quantitative relationships hard to derive. A better understanding of drug–surfactant interactions can be achieved from thermodynamic data analysis. One approach is to use ITC, wherein the enthalpy of interaction is measured directly when a solution of drug is titrated by a surfactant solution. ITC has been used in this way to study the interactions of salbutamol sulfate with Span® 85 and simvastatin with a number of surfactant micelles (sodium dodecyl sulfate, hexadecyl trimethylammonium bromide, sodium deoxycholate and polyoxyethylene 23 lauryl ether) [60]. From the results, a correlation between the free energy of transfer for the drug to each surfactant and the solubility enhancement of that surfactant was established. The results suggested that ITC screening of a range of surfactants against a poorly water-soluble drug might allow the selection of the best potential solubilizing surfactants.

Polymer–surfactant interactions

The development of polymer–surfactant formulations of acceptable blends requires methods for optimizing the physicochemical properties of the system. This can only be achieved through the controlled manipulation of the polymer–surfactant interactions [61–68]. A modern calorimetric technique such as ITC is one of the most sensitive techniques that permit the direct measurement of thermodynamic changes during polymer binding to the surfactant [69]. The interpretation of thermodynamic parameters variation leads to a determination of the type of noncovalent forces operating between the surfactant and the polymer, which is the starting point to the understanding of the mechanism and the theoretical description of polymer–surfactant interactions. Furthermore, the

FIGURE 2

Typical ITC data obtained from the micellization experiment of a surfactant molecule. The panel **(a)** shows exothermic heat released upon injection of the surfactant. The panel **(b)** shows integrated heat data, giving a sigmoidal micellization curve. The CMC is defined as the concentration where the first derivative of the curve b reaches a maximum **(c)**.

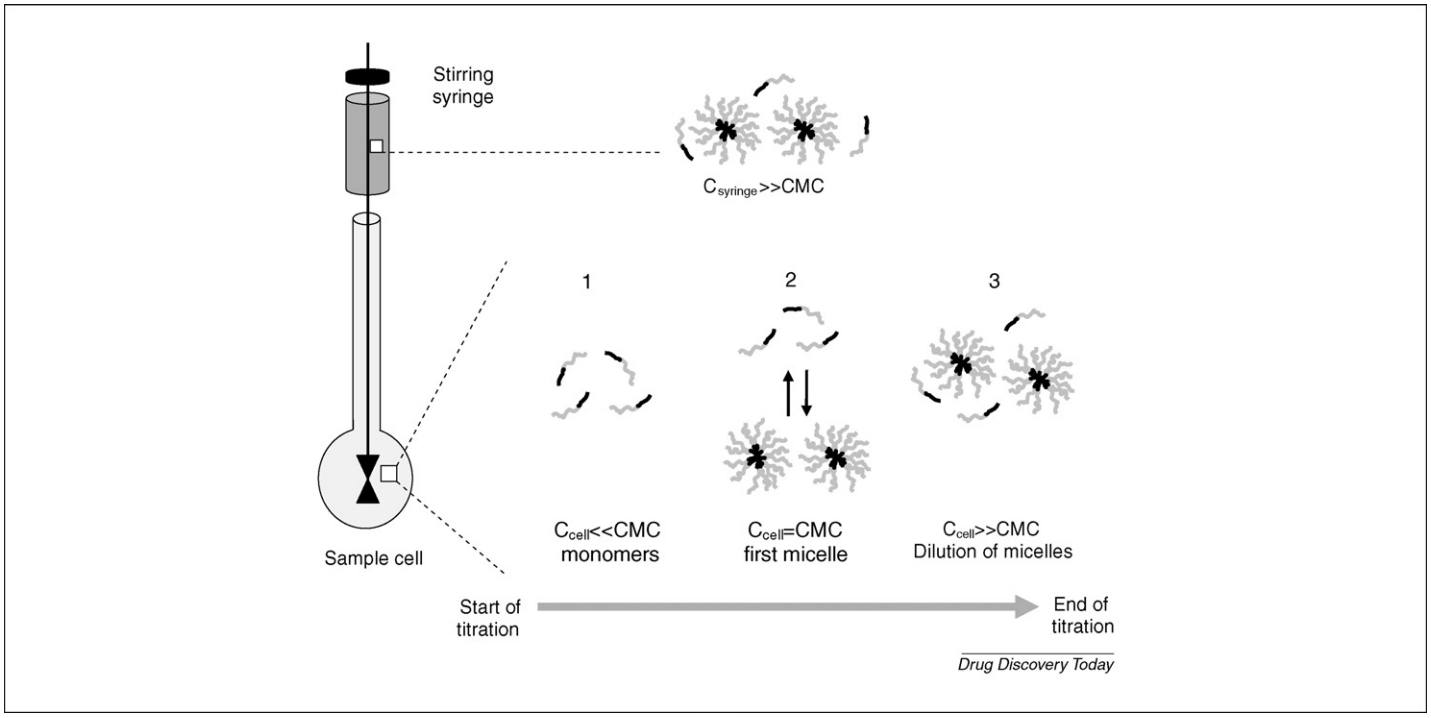


FIGURE 3

Schematic representation of the micellization experiment.

study of polymer–surfactant interaction of operating parameters variation such as temperature and additives leads to a clear knowledge of manufacturing conditions that are essential to control the polymer–surfactant binding.

In general, polymer–surfactant interactions can be divided into two broad categories: (i) charged polymers and oppositely charged surfactants and (ii) uncharged polymers and all types of surfactants.

- The first category of polymer–surfactant interactions concerns polymers and surfactants bearing opposite charges. For example, ITC was used to determine binding interactions in aqueous solution between cationic surfactant dodecyltrimethylammonium bromide and poly(acrylic acid) [64], methacrylic acid–ethyl acrylate copolymer [70] and binding of sodium dodecyl sulfate to a monodisperse poly(acrylic acid) [71], polyelectrolyte sodium alginate [72] and chitosan [73,74]. It was demonstrated from ITC experiments that binding interactions between polymers and surfactants bearing opposite charges, the electrostatic forces play an important role. Furthermore, in these experiments, an interesting phenomenon noted is that the polymer–surfactant complex precipitates when the amounts of positive and negative charges are equivalent, whereas such precipitates can be re-solubilized in excess amounts of surfactant [75,76].
- The second category of the polymer–surfactant interactions concerns neutral polymers like water-soluble cellulose derivatives including methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, hydroxypropylmethyl cellulose [78] or other polymers such as poly(vinyl pyrrolidone) [78,61,77], poly(vinyl methyl ether), poly(vinyl imidazole) [78], poly(propylene oxide), poly(ethylene glycol) [79], poly(ethylene oxide) [80], poly(propylene glycol) [81] and oxyphenylene–oxyethylene diblock copolymers [82,83]. Recently, the interaction between hydrophobically modified polymers (dextrin) and ionic surfactants was investigated using ITC [84]. For the studied complex systems such as hydrophobically modified polymers and surfactants interactions, besides important parameters that can be derived from calorimetric titration curves, such as critical concentrations and enthalpies of aggregation, the effects of hydrophobic side group concentrations on the interaction can be evaluated in detail [84]. Importantly, this work has shown that the aggregation behavior of the mixed systems depends on the molar ratio of surfactant to hydrophobic side group of polymers [84].

Protein–surfactant interactions

Surfactants play various roles upon interaction with proteins, such as protein refolding [85,86], unfolding [87], enzyme activation [88] and protein solubilization at the CMC level. Specifically, nonionic surfactants are habitually added to pharmaceutical formulations with the aim to prevent and/or minimize protein aggregation during fermentation, purification, freeze-drying, shipping and storage [89]. More specifically, nonionic surfactants can protect protein against surface-induced aggregation by binding to hydrophobic regions of the protein surface, and thus, decrease intermolecular interactions [90]. In these cases, the degree of protection can be maximized at the molar binding stoichiometry that can be determined by studying the interaction between a surfactant and a protein using ITC.

The protein–surfactant interactions may be advantageous in some cases whereas in other cases, the interactions may have a damaging effect on protein stability or enzyme activity. In this context, an understanding of the mechanisms involved in protein–surfactant interactions constitutes a rational strategy to optimize these applications. Recently, some research papers have

emerged, in which ITC has been used to obtain a protein–surfactant interaction isotherms. This implies titration of the protein with high surfactant concentrations. So far bovine serum albumin [91] and insulin [92] are usually selected as a model protein because their structure and physicochemical properties are well characterized. Interactions of cationic and anionic surfactants with lysozyme [93,94], gelatin [94] and cellulase [95] have also been investigated using ITC.

The proteins interactions with both nonionic and ionic surfactants showed complex interactions enthalpograms, which are rich in information, but challenging to interpret in terms of molecular events including contributions from one or more equilibria, like binding, conformational changes in the protein and micellization. For these reasons, the combination of calorimetric and structural data provides a unique possibility to make both molecular and quantitative interpretation of the processes involved in protein–surfactant interactions. This can be achieved by combining ITC to other techniques such as fluorescence spectroscopy [89,96,97], ultra-sensitive differential scanning calorimetry [91], ultraviolet-circular dichroism [92], turbidity [93], dynamic light scattering and fluorescence spectra measurements [51,52]. These works contribute to a better understanding of the forces involved in the surfactant–protein interaction. The results provide detailed information on the structure–property relationship of protein modulated by the oppositely charged surfactant.

Thermodynamic investigation of polyelectrolyte complexes

The spontaneous formation, of polyelectrolyte supramolecular structures as complexes and coacervates induced by the interactions of oppositely charged polyions, is a fundamental physicochemical phenomenon. This can be relevant not only to a number of biological processes such as protein transcription, antigen–antibody reactions or enzymatic channeling, but also to the design of delivery systems including micro- and nano-encapsulation processes, multilayers structures [98], the formation and stabilization of emulsions [99], the formation of gels [100], proteins delivery [101–103] and polycation complexes with nucleic acids or oligonucleotides as vectors in gene therapy [104]. The rapid and nearly athermal nature of electrostatic interaction makes it very difficult to examine and thermodynamic study of both bio-macromolecules binding interactions and synthetic polyelectrolyte aggregation needs techniques of high sensitivity and accuracy like ITC. This technique has been shown to be an effective tool for the thermodynamic study of both bio-macromolecules binding interactions and synthetic polyelectrolytes aggregation by the determination of equilibrium constants, stoichiometry and binding partners under defined experimental conditions [102].

It is well known that the formation of polyelectrolytes complexes takes place spontaneously when the total Gibbs free energy change ΔG decreases [101,105]. The value of ΔG is determined by a balance between entropic and enthalpic contributions.

Concerning the enthalpic contribution upon polyelectrolyte formation, in some cases, the heat of binding recorded by ITC displayed a complex pattern with the successive appearance of an exothermic and endothermic signal [106–110], and even of the appearance of a simultaneous exothermic–endothermic signal [110]. This can be associated with the (i) formation of polyelectrolyte complex upon binding, (ii) conformational change of the reactants, (iii) ionization of polar groups and (iv) interactions with the medium components. As a consequence, the calorimetric enthalpy change measured in an ITC experiment must be interpreted carefully, because the ΔH observed would represent the total of these enthalpies change for the process occurring in the sample cell.

Concerning entropy variation upon the polyelectrolyte formation, it was suggested that the process is mainly entropically driven through the release of water molecules [105] and of condensed counter-ions via the ion-exchange process [111–113]. In some cases, unfavorable entropic contributions were reported resulting from the decreased mobility of interacting polyions upon binding and, possibly, from the ordering of water at the complex interface [105].

Finally, by studying thermodynamics of polyelectrolytes interactions by increasing temperatures, it was shown that the interactions are accompanied by a positive heat capacity changes due to hydrophobic effects [114].

A critical analysis of thermodynamic data reported in many works shows that enthalpy or entropy contributions upon polyions interactions may be related to the density charge of polyelectrolytes. Indeed, it was shown that complex formation between weakly charged polyelectrolytes is driven by the negative enthalpy due to electrostatic attraction, with counter-ions release, when entropy plays only a minor role. On the contrary, complex formation between highly charged polyelectrolytes is driven by large counter-ions release entropy and opposed by a positive enthalpy change [101]. One strategy to detect the contribution of weak energy interactions, is for instance to vary the temperature and the pH [114] of interacting systems, to add different salt concentrations or chemical compounds to the medium of interaction [101].

Binding of oppositely charged drugs and polymers

Ionic interactions of the oppositely charged drugs and polymers have been reported to be a key factor of polyelectrolyte formation and stability as well as drug entrapment and release [115]. The high sensitivity of ITC was used to detect the effect of charge density, hydrophobicity and salt concentration upon the interaction between a positively charged drug (e.g. doxorubicin) with a negatively charged polymer (e.g. pluronic–polyacrylic acid copolymers) [116]. In that study, it was reported that interactions are governed primarily by electrostatic forces. The addition of salt weakens the interaction of drug and polymer by charge shielding effect between positive ionized amino groups on doxorubicin and oppositely charged polymer chains. However, at higher salt concentrations, the aggregation of doxorubicin is enhanced, whereas the affinity of the drug for polymer is decreased. In similar studies, ITC has been used to study the interaction of small molecules (aspartic acid and valine) to poly(*N*-isopropyl acrylamide) microgels as a function of temperature [117].

On the contrary, ITC can be used for the investigation of the interaction of drugs with living systems such as the cellular membrane surface and its components. As an example, ITC was combined to fluorescence spectroscopy for the determination of thermodynamics of the interaction of positively charged drug molecules (propranolol hydrochloride, tacrine and aminacrine), and the negatively charged polymers used as a cellular membrane model glycosaminoglycans (dextran sulfate, chondroitin sulfate and hyaluronic acid). The results highlight the importance of the

formation of drug–glycosaminoglycans complexes as a primary step for the drug delivery process into cell membranes [118].

Polyelectrolytes–proteins complexes

The investigation of polyelectrolytes–proteins complexes using ITC was used for (i) colloidal protein delivery system characterization, (ii) identification of manufacturing conditions in which reproducible and stable nanocomplexes are formed and (iii) improvement of entrapment, and sustain the release of the protein [119].

Typically, ITC was performed by titrating polymer solutions of known concentration and pH to protein solutions contained in the sample cell while stirring. As an example, the use of ITC for the thermodynamic investigation of the interaction of chitosan derivatives with bovine serum albumin [103] and the interaction of insulin with water-soluble amphiphilic polyesters containing a positively charged amine groups [102].

In the last example, the selfassembling nanocomplexes were formed immediately after being mixed and were characterized using ITC combined with other techniques such as photon correlation spectroscopy, laser Doppler anemometry, turbidimetry and atomic force microscopy. In the same study, it was demonstrated that the increase in the binding constant could be attained by two ways: (i) by increasing charge density with a higher degree of amine substitution and (ii) by introducing of short hydrophobic side chains, leading to a strong increase of association constant because of additional hydrophobic binding forces [102]. The influence of polymer structure on nanocomplex size could be observed when comparing polymers with the same degree of amine substitution but differing degrees of lactic acid grafting. The higher amount of lactic acid led to increasingly smaller complexes, possibly because of the influence of hydrophobic interactions on the complex structure [102].

The usefulness of ITC for the thermodynamic investigation of the selfassembly system composed of insulin and chitosan constitutes a second good example of the interaction between negatively charged drug and positively charged polymer. The enthalpy change of the titration between insulin and chitosan is partly because of the ionic interaction between both molecules. Part of the enthalpy variation may be because of conformational changes, ionization of polar groups and especially because of adsorption phenomena of insulin at the surface of the polymer carrier [120,121]. The conformational changes can be caused by ionic interactions and hydrogen bonds between amino-acid groups with charged side chains or hydrophobic interactions of amino-acid groups with nonpolar side chains [115].

Interactions of nucleic acids with multivalent cations

The transfer of nucleic acids into the cell is an important approach for biological and medical research and for potentially therapeutic applications. Under normal physiological conditions, nucleic acids have a high negative charge density that causes electrostatic repulsion with negative charges of cellular membranes. To overcome this transport barrier, charges of nucleic acids must be shielded, or even inversed to facilitate the cellular uptake by endocytosis. One of the several methods of nucleic acids delivery to cells is the use of polycations that can bind to polyanionic nucleic acids, facilitating their delivery process to the cell. It was demonstrated that the incorporation of nucleic acids into lipid vesicles, for example, cationic liposomes [122–124], cationic proteins, like histones and protamines, lipophilic peptides [125] or synthetic polymers [126–129,130], like polylysine, polyarginine, polyornithine, polybrene, polyethyleneimine, cobalt hexamine [131–133], spermidine [131], etc. may be effective intracellular nucleic acids delivery agents.

The nucleic acids–multivalent polycations complexes should be resistant during the storage, and against the competition of extracellular polyanions. They should be delivered to the cells releasing their nucleic acids cargo into the cells in a controlled way.

Even though a number of rigorous biophysical studies of nucleic acids–multivalent cations interactions and considerable progress have been made, the molecular mechanism of these interactions is not well understood and the formation of the complexes, their cellular entry and dissociation are not governed in a controlled manner. In this context, we need to answer the questions: What are the forces that drive the binding and conformational changes? In complex pharmaceutical formulations, what are the components that effectively interact with nucleic acids allowing the formulation efficiency? What are the key parameters that govern the efficiency of the biological effect? Facing these challenges, ITC was used to investigate the thermodynamics of nucleic acids interaction with multivalent cations and to understand the dynamics of the interaction.

In this section of the review, we give examples about the use of ITC to study DNA interaction with cationic surfactants. Typically, in these experiments, a micellar solution of cationic surfactant is injected into DNA solution contained in the sample cell. The thermal response signals showed that the interaction of DNA with cationic surfactants occurs when the cumulative concentration of surfactants in the sample cell reaches the critical aggregation concentration (CAC) and there is no detectable interaction below the CAC [134]. The CAC and the thermodynamic parameters for the aggregation process were obtained from the calorimetric titration curves. It was demonstrated from the resulting ITC titration curve that the aggregation process is generally spontaneous since the free energy of the interaction is negative [134,135]. Most of the thermodynamic studies revealed that the interaction of DNA with cationic surfactants is an entropy-driven process ($\Delta S_{agg} > 0$ and $T\,\Delta S_{agg} \gg |\Delta H_{agg}|$) where hydrophobic interaction of the surfactant molecules plays a key role in the formation of the DNA–cationic surfactants complex.

ITC was used for the investigation of the interaction of DNA with a series of alkylbenzyldimethylammonium chlorides [134], a series of gemini surfactants [135–137,46], cetylpyridinium chloride [138], functional dendritic polymers [139] and more recently trehalose click polymers [140].

Results obtained from ITC can be correlated to other techniques such as UV spectroscopy [134], fluorescence techniques [136], dynamic light scattering [134,136,140], surface tension measurements [134,136], conductivity measurements [137], gel electrophoresis [140], surface plasmon resonance [141] and transfection experiments [140]. Thanks to the correlation between the observed energetics variations obtained from ITC and results obtained from these techniques, the observed structures formed can be more fully understood and the mechanism of the interaction can be clearly revealed.

Probing of DNA interactions with small molecules

The interaction of small molecules with nucleic acids is currently the subject of intense studies, partly because of the need to develop novel agents for therapeutic, biotechnological and diagnostic purposes [142–147]. It concerns for example anticancer drugs [148–150] and antibiotics work by targeting DNA within the nucleus of cells [151].

Information obtained from ITC will be helpful to understand the interaction mechanism of small molecules with nucleic acids, and should be useful in the development of new therapeutic reagents [152–159]. Ligands with high-binding affinity and strong selectivity have potentially useful genomic-based therapeutic and diagnostic applications. Thus, understanding how both the drug and the DNA contribute to the overall stabilizing (and destabilizing) forces in complex formation is fundamental to the full characterization of these interactions.

Thanks to the correlation between the observed energetics variations obtained from ITC and three-dimensional structures of nucleic acids–drugs complexes from other techniques such as NMR and X-ray studies, the observed structures formed as well as the mechanism of the interaction can be more fully understood [149].

ITC for the characterization of nanoparticles

Despite the fact that nanoparticles offer several advantages as drug delivery systems, efficient nanoparticles formulations should combine a number of different properties such as efficient drug loading, controlled drug release, stealthiness and drug targeting. This can be achieved by the design of nanoparticles bearing different functionalities at their surface (Fig. 4), including for example

(i) Cyclodextrins which are able to increase the aqueous solubility of drugs [160],
(ii) Hydrophilic moieties are able to repulse proteins and avoid their adsorption such as poly(ethylene glycols) [161–163] and polysaccharides [164,165],
(iii) Ligands are able to recognize specific molecular structures at the target site [163,166].

In this context, an understanding of the biological effects of nanoparticles after their administration requires correct characterization of their surface properties and deep knowledge of rates, affinities and stoichiometries of the binding properties of biological molecules that associate with nanoparticles. In this last section of the review, the potential of the ITC for the characterization of different functionalities carried by polymeric nanoparticles as well as their interactions with living systems particularly, proteins was developed.

Characterization of the presence of cyclodextrins into nanoparticles

The encapsulation of hydrophobic drugs within nanoparticles results in a low drug loading efficiency, or a slow even incomplete release of the encapsulated drug. The incorporation of cyclodextrins into the nanoparticles should not only increase low drug loading but also allow the incorporation of ligand-terminated molecules thanks to the specific and strong interaction between

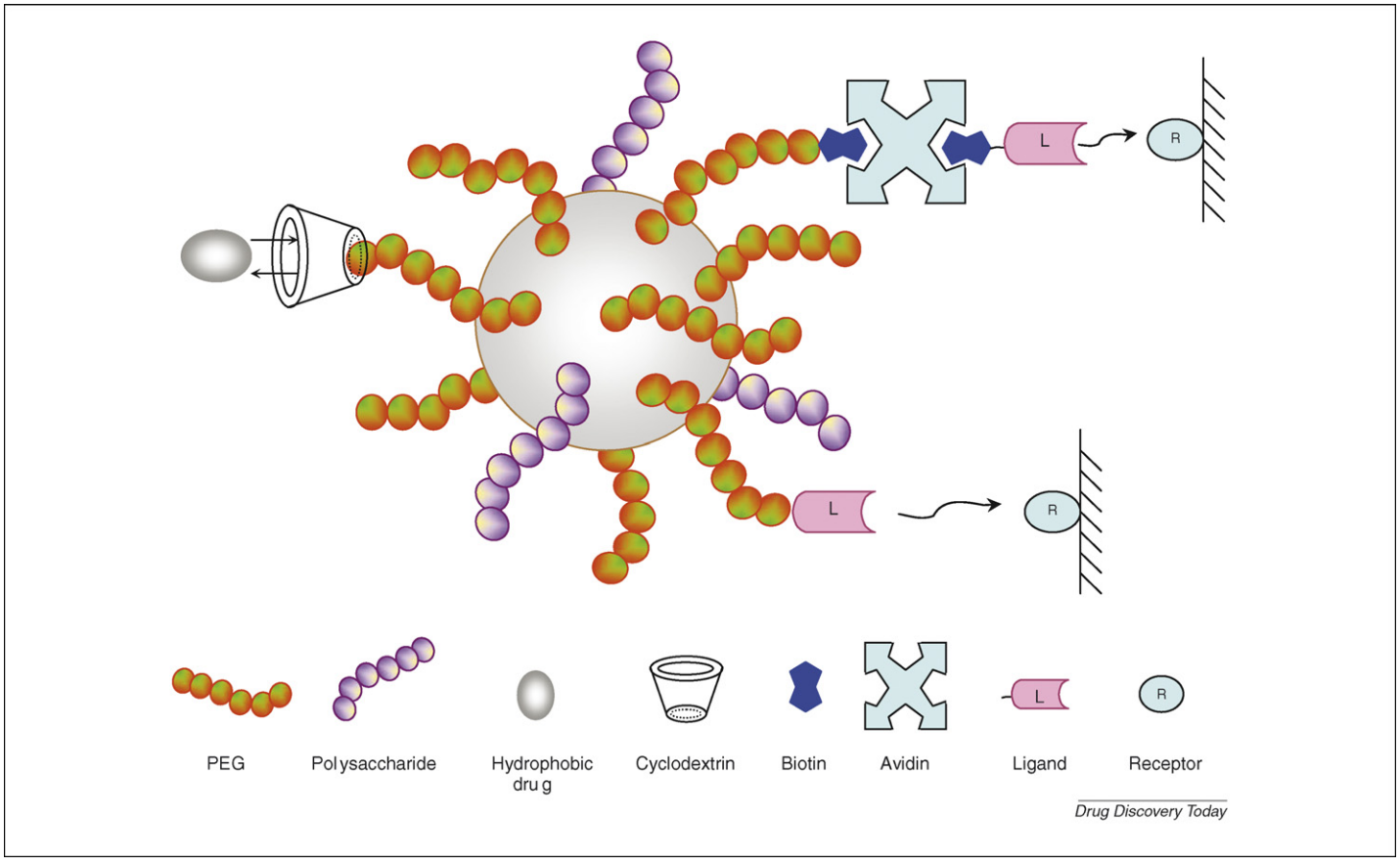


FIGURE 4

Schematic representation of different functionalities which can be attributed to nanoparticles.

hosts and cyclodextrins. For example, ITC was used with the aim to confirm the presence of the functionality of β-cyclodextrins at the surface of gold nanoparticles bearing surface-immobilized β-cyclodextrins hosts by their interaction with adamantyl-terminated guest molecules [160]. For this purpose, a solution of adamantyl-terminated guest molecules was injected into a suspension of nanoparticles bearing β-cyclodextrins. An exothermic signal was observed after each guest injection into the suspension, corresponding to the interaction between adamantyl-terminated guest molecules and β-cyclodextrins. After the fit of the integrated curve, the assembly was shown to be strong and specific by the addition of adamantyl-terminated guest molecules [160].

Biomimetic interaction of proteins with functionalized nanoparticles

As a result of their small size, the first generation of nanocarriers can be administered intravenously. However, in living systems, proteins associate with nanoparticles because of their hydrophobicity. It was demonstrated that nanoparticles are opsonized in the bloodstream after intravenous administration and cleared quickly by phagocytic cells [167]. To overcome these drawbacks, research has been focused on the modification of their surface by the coating of hydrophobic nanocarrier surfaces with hydrophilic flexible polymers, such as polysaccharides or poly(ethylene glycols). As a result, the intravenous injection of this second generation of nanoparticles induces a significant decrease in opsonization process. In the case of non-PEGylated nanoparticles, different proteins compete for the nanoparticle surface, leading to a protein corona that largely defines the biological identity of the nanoparticle. A deep understanding of the biological effects of nanoparticles requires knowledge of rates, affinities and stoichiometries of the binding properties of proteins that associate with the nanoparticles. However, the isolation and the identification of particle-associated proteins, which represent a fundamental prerequisite for nanobiology, nanomedicine and nanotoxicology, is not a simple task.

A central methodological problem to isolate and identify particle-associated proteins is to separate free protein from protein bound to nanoparticles. The challenge is to use methods that do not disrupt the protein–particle complex or induce additional protein binding based on established techniques. An original approach based on ITC can be used to assess the stoichiometry and affinity of protein binding combined to surface plasmon resonance studies allow additional data on protein association/dissociation from nanoparticles [168]. The results indicate that many proteins form transient complexes with nanoparticles and that there is a clear dependence of the binding and dissociation parameters on protein type and the nanoparticle surface characteristics. The resulting corona then effectively constitutes the new nanoparticle surface, whereas its shape and size also may play an important role in its interactions with cell surfaces.

The total protein concentration in bodily fluids, especially in intracellular environments, represents several thousand different proteins spanning a wide range of concentrations that are able to interact with nanoparticles. As a result, there will be competition between the proteins for the available nanoparticle surface area in a typical biological environment. Human serum albumin and fibrinogen may adsorb on the particle surface at short times but will subsequently be displaced by lower abundance proteins with higher affinity and slower kinetics. By contrast, when the available nanoparticle surface area is in excess over the total available protein, lower affinity proteins such as albumin also may be found in isolation experiments. ITC can be used to identify a set of associated proteins that more closely reflects the situation *in vivo*. This will require the protein mixture (e.g. plasma) to be in excess over the available particle surface area, and that the particle concentration used reflects as far as possible the true biological situation [168].

Probing nucleic acids interactions with gold nanoparticles

DNA-based nanotechnology has generated interest in a number of applications owing to the specificity, programmability and reproducibility of DNA interaction with nanoparticles. Gold nanoparticles modified with DNA are useful in diverse fields such as DNA chips [169], DNA sensors [170] and interestingly, DNA delivery and targeting. Similar applications are envisaged for the DNA analog, peptide nucleic acids (PNAs), wherein the sugar–phosphate backbone is replaced by a polyamide backbone [171]. The remarkable utility and versatility of such systems is attributed to the nature of the interaction of DNA with gold nanoparticles, which in turn is determined by the differential affinity of the nucleobases (adenine, guanine, thymine and cytosine), nucleosides and oligonucleotide sequences to the gold nanoparticles [172]. Previous studies on understanding the nature of interaction of nucleobases with gold nanoparticles have made use of various spectroscopic techniques such as surface-enhanced Raman spectroscopy [173], Fourier transform infrared [174] and reflection absorption infrared spectroscopies [175] which are clearly indirect methods for the estimation of the strength of the above-mentioned interactions.

ITC may be used to directly observe the energetics of interaction of the DNA bases as well as the corresponding PNA base monomers with gold nanoparticles [176]. Results showed that the strengths of interaction of the nucleobases and PNA analogs with gold nanoparticles might be evaluated on the basis of the exothermicity during the initial stages of interaction. In that study, it was demonstrated that the strength of interaction of the nucleobases–PNA analogs decreases in the order $C > G > A > T$. It is interesting to note that the PNA monomers bind much more strongly to the gold nanoparticle than the DNA bases and that a concentration ten times smaller than that of the DNA bases is enough to bring about the saturation of the gold nanoparticle surface [176].

Targeting ligands

The active targeting of nanoparticles to organs other than liver and spleen (reached by passive targeting) can be achieved with nanoparticles carrying ligands, such as antibodies or peptides containing cell-binding sequences that are able to recognize and bind specifically to target site. To prepare nanoparticles with targeting moieties on their surface, several methods have been explored, including the covalent attachment of ligands to surfaces based on molecular recognition between avidin and biotin or adsorption of antibodies or antigens onto the particle surface.

One of the major challenges faced is the low amount of antigen associated with the nanoparticle systems resulting on a difficult access to the quantification using classical techniques. One solution to this problem is the use of ITC to investigate the interaction of the ligand immobilized at the surface of nanoparticles to the target site.

As an example, the adsorption of tetanus toxoid as a cationic antigen at the negatively charged surface of blank nanoparticles composed of sulfobutyl-poly(vinyl alcohol)-*g*-poly(lactic-*co*-glycolic acid) copolymers with a core–corona structure. ITC results combined to zeta-potential measurements showed that the nanoparticle surface properties dictated the adsorption efficiency of tetanus toxoid onto the nanoparticles, and as expected, the maximal loading rate was achieved at the highest degrees of sulfobutyl substitution, which implies that the nature of the tetanus toxoid–nanoparticle interactions was primarily electrostatic [177].

Conclusion

In conclusion, the power of ITC derives from the universality of the technique since every reaction generates or absorbs heat, so every reaction can in principle be studied by high sensitivity titration calorimetry. This technique provides a widely applicable method for monitoring molecular recognition in a label-free manner and so is a potentially attractive technology for use in drug discovery, drug delivery and pharmaceutical formulations.

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