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Potential applications of microcalorimetry for the study of physical processes in pharmaceuticals

Simon Gaisford[1], Graham Buckton[*]

Department of Pharmaceutics, School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX, UK

Abstract

In calorimetry, the heat-flow to or from a sample is measured as a function of time (isothermal calorimetry) or temperature (scanning calorimetry). The technique is not dependent on the physical form of the sample and is usually non-destructive (exceptions include temperature-induced irreversible phase transitions and thermal decomposition). The inherent sensitivity of modern instruments allows measurements on the micro-Watt scale. Calorimetry is highly suited to the study of pharmaceutical systems because small sample masses are usually required and the technique is very sensitive to changes induced by, for instance, formulation or processing. It is the purpose of this review to show applications of both isothermal and scanning calorimetry in the field of physical and bio-physical pharmacy. Potential applications include studies of physical stability, excipient compatibility, chemical stability and the study of the potential interactions of and between macromolecules such as lipids, surfactants, and nucleic acids.

Keywords: Isothermal microcalorimetry; High-sensitivity differential scanning calorimetry; Titration calorimetry; Excipient compatibility; Drug stability

1. Introduction

Calorimetry is merely the measurement of heat (power, q, in W) with time (t) or temperature (T). Microcalorimetry is the measurement of such heats on a micro-Watt scale (note that the prefix micro does not, in this case, refer to the sample size). Isothermal microcalorimeters measure power as a function of time. Scanning calorimeters measure power as a function of temperature. The technique offers some qualities that distinguish it from many other analytical techniques. Principally, the instrument does not intrude upon the sample (although, of course, in scanning calorimeters it is possible that some irreversible phase transitions may be induced). Neither is it dependent on the physical nature of the subject under study; the sample can be a solid, liquid or gas or any combination of the three. This allows the direct investigation of a wide range of systems, the only constraint being sample size. The heat-flows associated with all the reactions that are occurring simultaneously within the sample are recorded. This non-specificity has both benefits and drawbacks; it allows many complex reactions, which are outside the scope of other analytical tools, to be studied. However, it also means that poor sample preparation can lead to erroneous heat-flow signals and it may be that the heat-flow signal is influenced greatly by an effect other than that which is the intended subject of the study. Consequently, the use of microcalorimetry offers much potential, but there is a need to balance good experimental design with careful data interpretation. Calorimetry is highly suited to the study of pharmaceutical systems because

[*] Corresponding author.

[1] Present address: School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield, W. Yorkshire HD1 3DH, UK.

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the technique is very sensitive to changes induced by, for instance, formulation or processing. It is the intent of this paper to give an overview of some of the potential applications of microcalorimetry to the characterisation of pharmaceutical systems that are undergoing physical changes.

2. Principles of calorimetry

2.1. Isothermal microcalorimetry

In isothermal microcalorimetry, any heat produced or absorbed by a sample is, ideally, completely exchanged with a surrounding heat-sink, maintaining the sample at a constant temperature. Usually, the reference chamber is loaded with an inert material, of similar heat capacity and in a similar quantity to the sample, and the heat-flow to or from the sample side is compared with the heat-flow (zero normally) to or from the reference side (differential mode). A modern heat conduction, isothermal microcalorimeter is capable of maintaining a baseline of $\pm 0.1\ \mu W$ with a temperature stability of $\pm 0.0001^{\circ}C$. It conducts heat to a heat-sink via semiconducting Peltier elements. The design of such an instrument has been described in detail previously [1]. The 2277 thermal activity monitor (TAM) by thermometric (Järfälla, Sweden) is a typical instrument and consists of a thermostatted water bath surrounding a calorimetric channel. A channel comprises a reference and a sample side and data are obtained as a differential response between sample and reference. Consequently, most of the noise arising from temperature fluctuations is removed when the reference data are subtracted. The instrument should be calibrated periodically by an electrical substitution method, although recently the issue of chemical test reactions has been discussed [2,3]. In principle chemical test reactions (such as the imidazole-catalysed hydrolysis of triacetin) offer a more realistic test of the operating performance of a calorimeter.

2.2. High-sensitivity differential scanning calorimetry

In differential scanning calorimetry (DSC) the power supplied to a sample to raise it in temperature at a constant rate is measured and is compared with that supplied to an inert reference material undergoing the same temperature programme. DSC is a technique used throughout the pharmaceutical industry, typically being employed for physical characterisation of materials and, occasionally, for excipient compatibility testing or stability screening. DSC instruments can operate at temperature scan rates of $200^{\circ}C\ min^{-1}$ or higher (although between 5 and $20^{\circ}C\ min^{-1}$ is more usual) [4], but this had led to instruments with a sensitivity, in practice, of ± 1 mW. Coupled with a small sample size (typically 5–20 mg) this relative lack of sensitivity has precluded the use of DSC for studying, for example, reactions in dilute solution, reactions of macromolecules and reactions that occur with a small change in reaction enthalpy, ΔH.

The need to study biological molecules in their native state led to the commercial availability, during the past 20 years, of a range of DSC instruments with a much greater calorimetric sensitivity [4–7]. Such instruments (notionally referred to as high-sensitivity DSC, although the terms used by individual manufacturers vary) have a calorimetric sensitivity of $\pm 0.5\ \mu W$, mainly because they hold a much greater amount of sample (0.5–1 ml). They operate at relatively slow scan rates ($0–2^{\circ}C\ min^{-1}$) to allow the large sample to follow the temperature programme. Table 1 contains the details of a number of commercially available HSDSC instruments. The increase in both sensitivity and sample size over standard DSC instruments mean that HSDSC offers a wider range of pharmaceutical applications than standard DSC. Typical examples include the denaturation of proteins, phase changes in lipid bilayers, phase transitions in dilute polymer solutions, changes in structure of creams and emulsions and testing excipient compatibility.

3. Pharmaceutical applications of microcalorimetry

3.1. Vapour sorption

The study of interactions between, and reactions induced by, the presence of water in a sample is an important consideration for product stability. This is because water may be detrimental to a material in

Table 1
Comparison of the performance characteristics of a range of commercial HSDSC instruments

Instrument	Micro DSC III	Micro DSC VII	CSC 6100 nano-II DSC	CSC 4100	B-900	VP-DSC
Manufacturer or supplier	Setaram, 7 rue de l'Oratoire, F-69300 Caluire, France	Setaram, 7 rue de l'Oratoire, F-69300 Caluire, France	Calorimetry Sciences Corporation, 515 East 1860 South, P.O. Box 799, Provo, UT 84603-0799, USA	Calorimetry Sciences Corporation, 515 East 1860 South, P.O. Box 799, Provo, UT 84603-0799, USA	Sceres, 16 rue de Chartres, F-91400, Orsay, France	Microcal Inc. 22 Industrial Drive East, Northampton, MA, USA
Type	Heat flux	Heat flux	Power compensation	Heat flux	Power compensation	Power compensation
Furnace						
Scan modes	Heating, cooling, isothermal	Heating, cooling, isothermal	Heating, cooling	Heating, cooling, isothermal	Heating, cooling, isothermal	Heating, cooling, (isothermal titration calorimeter (ITC) also available)
Temperature range	−20 to 120°C	−45 to 120°C	−10 to 130°C (160°C optional)	−40 to 110°C (−40 to 200°C optional)	−190 to 850°C	−10 to 130°C
Scan rates	0.001–1.2°C min^{-1}	0.001–1.2°C min^{-1}	0–2°C min^{-1}	0–2°C min^{-1}	0.01–15°C min^{-1}	Maximum 1.5°C min^{-1} heating or maximum 1°C min^{-1} of cooling
Detection limit (μW)	±0.2–2	±1–5	±0.75	±1	±0.1	±0.35
Sample holder						
Construction	Hastelloy C	Hastelloy C276	24-K Gold	Hastelloy C (tantalum or titanium optional)	Steel, Teflon, Pt, glass, Hastelloy or other	Tantalum
Volume (ml)	1	1	0.33	1	1.5	0.5
Type	Batch or flow through cell	Batch or flow through cell	Fixed cell	Batch cell (holds up to three cells)	Batch or flow through cell	Fixed cell
Applications	Biological and pharmaceutical applications, protein denaturation	Foodstuffs and pharmaceuticals liquid crystal transitions, bacterial growth	Behaviour of biopolymers in solution, lipid membrane structure, ligand interactions	Cell metabolism, material stability, high throughput screening	Phase transitions and chemical reactions, pyrolysis, specific heats	Protein denaturation, gel transitions, melting of lipid bilayers

several ways. For instance, water may induce a hydrolysis reaction, cause an amorphous sample to recrystallise by lowering its glass transition (T_g) temperature, cause deliquescence of crystals, allow the formation of hydrates or act as an intermediary between two solid components. The relative ease with which such interactions can be studied has been made easier by the commercial availability of perfusion apparatus that allows the introduction of water vapour directly into the sample ampoule. Such apparatus allows the relative humidity (RH) of a gas flowing over a sample to be varied as a function of time.

An alternative, but related, experiment is to study a sample under conditions of a specific RH, by placing a small glass tube (a Durham tube or hydrostat), containing a small quantity of a saturated salt solution, directly within the ampoule. Saturated salt solutions maintain a constant RH at a specific temperature. Table 2 lists the RH obtainable at different temperatures with various saturated salt solutions [8]. The ''mini-hygrostat'' method gives rise to the generation and use of humidity within the measuring site of the calorimeter, hence much (but not all) of the wetting response for the sample is matched by the (almost) equal and opposite response for the generation of the humid air.[2] Consequently, the ''mini-hygrostat'' can be very useful for measurements in which a sample changes subsequent to humidity, whereas the gas flow method is well suited to measurement of wetting (and possibly subsequent changes if they are large enough to be visible in the presence of a large wetting response).

Studies of the interaction of materials with water vapour are very well suited to the study of amorphous contents of powders. Many techniques, such as milling, grinding or drying, used in drug manufacture induce the formation of amorphous regions in solid drugs and excipients. Above the glass transition temperature, amorphous materials will tend to crystallise rapidly. Crystallisation is accompanied by a change in heat capacity and can therefore be followed using microcalorimetry. Water acts as a plasticiser, lowering the T_g of the material, allowing crystallisation to be investigated at room temperature. The heat output of crystallisation is directly proportional to the amorphous content of the material. It has been claimed that it is possible to determine amorphous contents to $\pm 0.5\%$ using microcalorimetry compared with a detection limit of 10% with conventional techniques such as X-ray diffraction [9]. Examples of pharmaceutical materials that have been investigated using this technique include the sorption of water (or organic) vapour into amorphous cefditoren pivoxil [10], onto lactose [11,12] and onto griseofulvin [13].

Table 2
The relative humidities (RH) obtained at various temperatures using saturated salt solutions [8]

Salt	Temperature (°C)		
	25	35	45
LiCl	11.3	11.2	11.2
$MgCl_2$	32.8	32.0	31.1
NaBr	57.5	54.0	52.0
NaCl	75.3	74.8	74.7
KCl	84.3	82.9	81.7

Buckton and Darcy [11] have reviewed the use of hydrostats to study the response of amorphous lactose to changes in RH. In these experiments, amorphous lactose was sealed in a glass ampoule with a hydrostat containing saturated NaCl solution. A typical response is shown in Fig. 1 [14]. The data reflect the absorption of water by the sample, crystallisation and subsequent water expulsion. The trace can be seen to show two distinct regions; a small initial response followed by a large peak. It is possible to delay the onset of the second peak by either increasing the sample mass or decreasing the RH. Decreasing the RH below a critical point will lead to no crystallisation within a measurable time.

The first, smaller peak is thought to be associated with the evaporation of the salt solution and wetting of the sample. The shape of this peak varies considerably with different salt solutions. As such, care must be taken when choosing a reference for this type of experiment. It is possible to use an inert blank or a

[2] The generation and use of humidity in the ''mini-hygrostat'' means that both processes are measured by the calorimeter, giving rise to a very different response from gas-flow experiments, where the humidity is generated remotely from the measuring site. However, the generation and use of humidity from a salt solution does not give rise to an exact balance of exotherms and endotherms. It has been reported elsewhere that different salt solutions give different blank responses for example, due to the complex nature and kinetics of the processes, involving evaporation and changes in solution salt concentration.

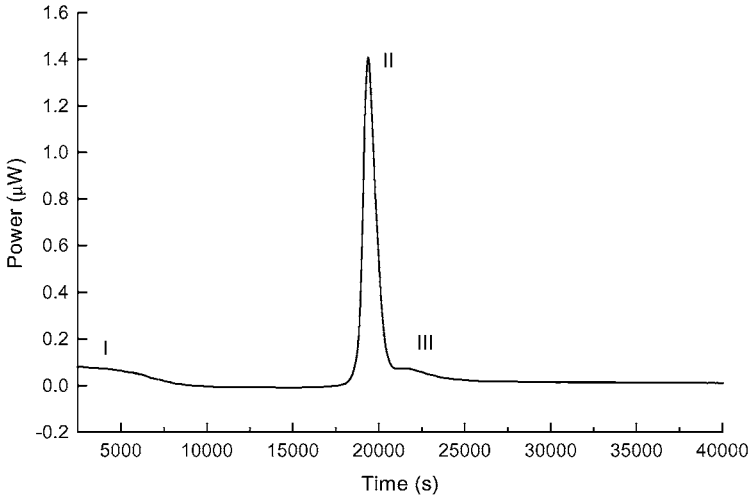

Fig. 1. Power–time trace of 100% amorphous lactose exposed to 75% RH [14].

reference cell containing an equivalent salt solution as that in the sample cell and it should be stated explicitly which type of reference was used. It has been suggested [15] that another contribution to the small signal is partial collapse in the sample. In fact, a sample collapsed by exposure to 50% RH showed no small peak. It seems likely that the initial small response is a combination of all these factors.

The second, larger peak corresponds to crystallisation of the sample. This can be demonstrated by showing that the sample is amorphous before the transition and crystalline afterwards using DSC. The peak is extremely sharp and the heat-flow decays rapidly to zero, although the response is not limited by the time constant of the instrument. Again, this signal is more complex than one might imagine, comprising contributions from crystallisation of the sample, expulsion of the absorbed water and condensation of that water back into the salt solution reservoir.

For lactose samples with lower amorphous contents, the large peak is often observed to be split into two separate regions, referred to as parts II and III (part I being the small initial response). It has been suggested that part II corresponds to crystallisation and part III corresponds to mutarotation of α-lactose to β-lactose [16]. Calculation of the crystallisation enthalpy using the area under the part II curve gives a value of around 32 J g^{-1} whereas that calculated using both parts II and III gives a value around 48 J g^{-1}. It should be noted that two distinct peaks during crystallisation might be observed for samples that do not exhibit mutarotation. A good example of this is provided by the crystallisation of salbutamol sulphate [17], where a distinct endothermic is seen in the crystallisation exotherm (before the peak reaches a maximum). Since the contribution of mutarotation to the overall response is not yet known, it cannot be certain as to which is the best way to analyse the lactose data. However, the evidence from salbutamol sulfate crystallisation makes it likely that peaks II and III are both part of the crystallisation response.

The crystallisation response is reduced in size because of the endothermic signal associated with water desorption. Recently, it has been shown that the measured calorimetric crystallisation peak plus the enthalpy of vaporisation for an estimate of the amount of water desorbed (i.e. the quantity of water needed to plasticise T_g below T minus the quantity of water which is retained as a hydrate) approximates to the combined endotherms for hydrate loss and melting which are measured in a DSC for the crystallised lactose [18]. This indicates that the contributions within the saturated salt solution are relatively small during the crystallisation response. Buckton and Darcy [18] concluded that since the net exotherm recorded in the microcalorimeter was the same at each RH studied at 25°C, the water desorption must have been similar at each RH. This means that the

amount of water sorbed at each RH was the minimum required to cause rapid crystallisation. This situation would arise if the supply of water vapour were slow, because of slow diffusion in the cell and the small surface area of the saturated salt solution. It has been shown that if the supply of water vapour is rapid, then amorphous lactose will equilibrate to a different water load at different RHs prior to crystallisation. It was fortunate that the experimental design in the microcalorimeter resulted in a balance of kinetics for which the area under the curve at each RH was essentially identical for crystallisation at 25°C. This situation held true for both amorphous lactose and amorphous salbutamol sulfate [17]. It is important to realise that the calorimetric response for crystallisation contains this substantial balance of exothermic and endothermic contributions. If the data are to be used quantitatively then the effects of changes in environmental conditions must be understood and any comparison between data at different temperatures and humidities should be undertaken with great care.

Aso et al. [19] have studied the physical stability of amorphous nifedipine using isothermal microcalorimetry and report similar responses for the net crystallisation at temperatures between 50 and 60°C over a range of relative humidities.

3.2. Pharmaceutical stability

One of the biggest obstacles in the widespread use of microcalorimetry to investigate pharmaceutical stability is the difficulty in analysing the data quantitatively. This problem is particularly acute for drug stability, which often involves solid phases, although microcalorimetry can play an important role in the development of solid-state drugs [20] and in determining long-term stability [21,22]. Nevertheless, many drugs have been subjected to microcalorimetric investigation including, for instance, aspirin [23], cephalosporins [24], lovastatin [25], meclofenoxate hydrochloride [26] and ascorbic acid [27,28].

Isothermal microcalorimetry has been used in the investigation of the oxidation of L-ascorbic acid in aqueous solution [27,28]. Ascorbic acid oxidises reversibly in aqueous solution forming dehydroascorbic acid, which is subsequently irreversibly hydrolysed to give diketogluonic acid. The rate of the reaction is affected by a number of factors including pH, oxygen concentration, ascorbic acid concentration, the presence of metal ions and the presence of anti-oxidants. Using the microcalorimeter it was possible to study this oxidation reaction under varying conditions and determine the effects of altering each of the factors.

Angberg et al. [27] noted that the heat-flows measured for solutions of ascorbic acid in pH 4.9 and 3.9 buffers were greater for those samples that were prepared with an air-space in the ampoule compared with those that were not. Furthermore, if the solution was purged with nitrogen prior to loading, the heat-flow dropped nearly to zero. Willson et al. [28] noted, by observing a linear ln(power) versus time plot, that the oxidation was first-order with respect to oxygen concentration. Both studies suggested that the oxygen in the ampoule is exhausted after 3–4 h and the measured heat-flow after this time falls to zero.

The measured heat-flow increased with increasing ascorbic acid concentration up to a certain concentration whereupon further increases did not increase the power response, Angberg et al. [27]. It was presumed the reaction became limited by the oxygen concentration at higher acid concentrations. Willson et al. [28] calculated the rate constants for the oxidation at varying ascorbic acid concentrations and found they were identical, concluding that the oxidation rate is independent of acid concentration. The presence of metal ions (copper or iron, for example) is known to affect the oxidation rate of compounds in solution and is difficult to control, because only trace amounts are required to catalyse the reaction. Both authors conducted experiments in the presence of EDTA, a metal chelating ligand and observed that the measured heat-flows fell substantially compared with samples run in the absence of the metal binder.

Otsuka et al. [26] investigated the stability of both meclofenoxate hydrochloride (MF) and DL-α-tocopherol (TP). MF hydrolyses in aqueous solution while TP oxides in air, the drugs providing good examples of two of the main processes by which pharmaceuticals are degraded. The hydrolysis of MF was investigated in aqueous solutions buffered to pH 6.4 and 2.9 and plots of ln(power) versus time gave linear relationships, indicating the degradation followed first-order kinetics. The rate constants at the two pHs were determined to be equal to 1.14×10^{-4} and $9.7 \times 10^{-7}\ s^{-1}$, respectively. These values concurred

with results generated from HPLC data of 1.29×10^{-4} and 9×10^{-7} s^{-1}. Samples of TP (a slightly viscous liquid) were placed in glass ampoules that were left open to the atmosphere in ovens at 50, 40, 30 and 23°C for varying lengths of time. Each sample was then capped before being placed in the microcalorimeter. Equivalent samples were analysed using HPLC. First-order rate constants for the samples were determined at each temperature. An Arrhenius plot of the data revealed a linear correlation and an excellent agreement between the HPLC and microcalorimetric data.

3.3. Excipient compatibility

Excipient compatibility is important when considering drug stability. All drugs are formulated with a range of excipients such as binders, disintegrants, fillers, lubricants, etc. It is important that the drug does not interact with any of the excipients in a way that is likely to reduce its efficacy. A simple methodology for testing binary mixtures in a microcalorimeter has recently been reported [29]. The thermal behaviour of the drug alone and the excipient alone are recorded and compared with that determined for a binary drug–excipient mixture. Any unexpected heat-flows recorded in the drug–excipient mixtures indicate a possible interaction. The method can be used with a wide range of drugs and excipients and there is therefore no associated method development required for each test subject as there is with, for instance, HPLC analysis [20]. Another advantage is that the experiment requires just 15 h to complete. Similar studies have been published by Selzer et al. [30], whereby the interactions between a solid drug and a range of excipients, including potato starch, α-lactose-monohydrate, microcrystalline cellulose (MCC) and talc were studied, and by Schmitt [31] using water slurries instead of humidified samples.

Wissing et al. [32] used HSDSC to study excipient compatibility in binary mixtures of aspirin/lactose and aspirin/magnesium stearate. Fig. 2 shows the thermogram recorded for a 1:1 aspirin:lactose mixture as it was raised in temperature in 5°C steps. It is important to note in these data that the instrument is operated principally in the isothermal mode. Temperature scanning was used to raise the sample temperature between isothermal phases (the advantage of this approach over conventional isothermal microcalorimetry is one of time — it takes at least a day to change the temperature of an instrument that utilises a large water bath). The endothermic and exothermic signals that are observed arise from the temperature lag in the sample as the system changes between isothermal and scanning modes. There is no deviation from baseline during each isothermal step once thermal equilibration has been achieved and it was concluded that the two

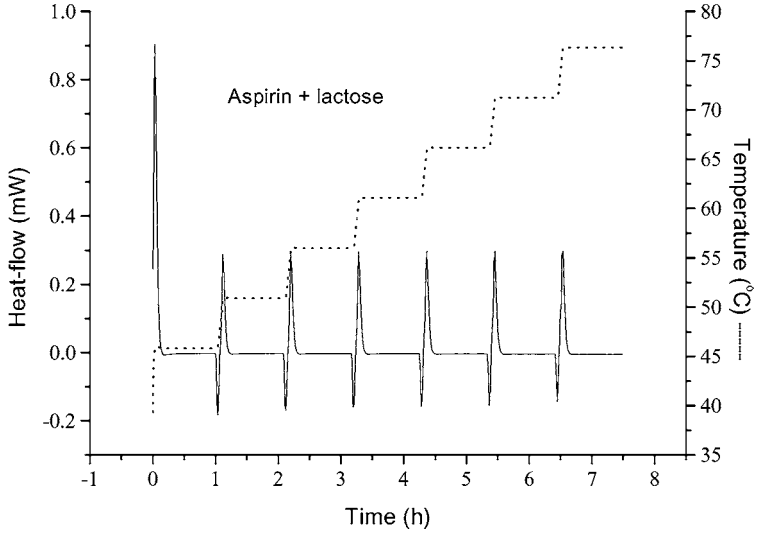

Fig. 2. HSDSC trace for a binary mixture of aspirin and lactose ramped in 5°C steps [32].

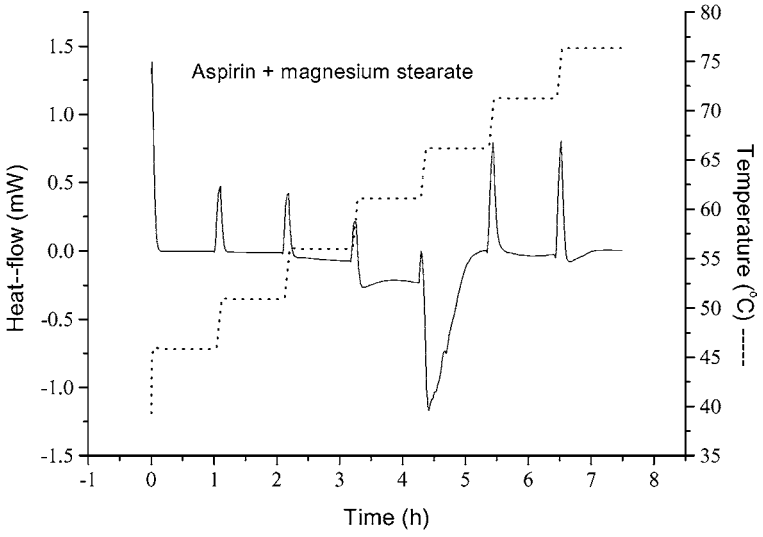

Fig. 3. HSDSC trace for a binary mixture of aspirin and magnesium stearate ramped in 5°C steps [32].

components did not interact. Fig. 3 shows the thermogram recorded for a 1:1 aspirin:magnesium stearate mixture. In this case endothermic signals can be observed during isothermal stages above 55°C, indicating that interactions have occurred.

HSDSC has also been used to study interactions between pharmaceutical preparations and liquid media. For instance, the pro-drug cefuroxime axetil is formulated by encapsulation into fatty acid microspheres. It has been observed that the rate of drug release from the fatty acid matrix is dependent upon, among other factors, the pH and ionic composition of the dissolution medium [33]. For instance, maximum drug release rates are obtained in pH 7.0 or higher buffers that contain either sodium or potassium ions, and virtually no release is observed in pH 5.9 or lower buffers. This effect has been investigated using HSDSC by adding a sample of encapsulated drug to buffer in the calorimeter cell, allowing the system to interact for 1 h and then subjecting the sample to a temperature ramp. It was noted that traces for the pure material exposed to water and pH 5.9 buffer were identical, while those for samples exposed to pH 7.0 and 8.0 buffers exhibited multiple endotherms. While the mechanism of drug release from the wax is unclear, the HSDSC experiments clearly demonstrate that an interaction has occurred in the higher pH buffers.

3.4. Polymer interactions

HSDSC has been employed to investigate phase changes in dilute polymer solutions as well as interactions between different polymers that are present in semisolid or solution phase formulations. For instance, Beezer et al. [34] used HSDSC to study the aggregation of dilute solutions of 27 members of the poloxamer series at 5 mg ml^{-1}. It was observed that as the temperature of a particular sample was increased a sharp, thermally reversible phase transition was observed. This transition has been ascribed to micellisation. The driving force of the transition is thought to be the dehydration and subsequent aggregation of the hydrophobic PPO moieties present in these polymers with an increase in temperature.

Gaisford et al. [35,36] studied the solution phase behaviour of binary mixtures of poloxamers using HSDSC. In this work, binary mixtures were prepared of poloxamers that had related compositions; either the two polymers shared the same percentage of PEO or they shared the same number of PPO groups. In most cases, one phase transition was observed. The transition temperature was the same as that observed previously for the poloxamer in the pair with the lower phase transition temperature [34,35]. This indicated that the aggregation of one poloxamer caused the premature aggregation of the second, leading to mixed

aggregates and the observation of a single endothermic. This was termed co-operative aggregation. In a few cases, the two polymers were observed to behave independently, giving rise to two endotherms. Such behaviour was seen in mixtures of the polymers P217, P237 and P407, poloxamers that are commonly found in pharmaceutical preparations.

The polyoxyethylene alkyl ether surfactants have also been the subject of investigation by HSDSC [37]. Solutions were prepared by heating the polymers in pH 7.2, 30 mM phosphate buffered saline to 80°C followed by 1 h in a sonicator bath at 30°C at concentrations of 3 and 15 mg ml^{-1}. When subjected to a temperature ramp, the polymer solutions were seen to undergo pre- and main-transitions. The pre-transition involved a large number of co-operative units and was thought to involve a large micelle or a crystalline or liquid crystalline region. When the polymers were studied in the solid state using conventional DSC, the pre-transition was still seen, supporting the idea that the pre-transition may relate to a solid-state property which endures in the liquid dispersion [37]. The main transition was ascribed to a change in the polymer structure from a gel phase to a micellar phase.

Gaisford et al. [38] used isothermal microcalorimetry as a novel technique for studying the swelling of PEG-based hydrogels in water. In these experiments, a segment of dry hydrogel (xerogel) was immersed in water (1 ml) and the heat response from swelling recorded. A typical trace is shown in Fig. 4, which shows the thermal response of samples stored at 21 and 55°C. A two-phase process was observed, which the authors ascribed to the hydration of the polymer core and subsequent relaxation of the polymeric network. The break point time between the two processes was observed to reduce with an increase in storage temperature.

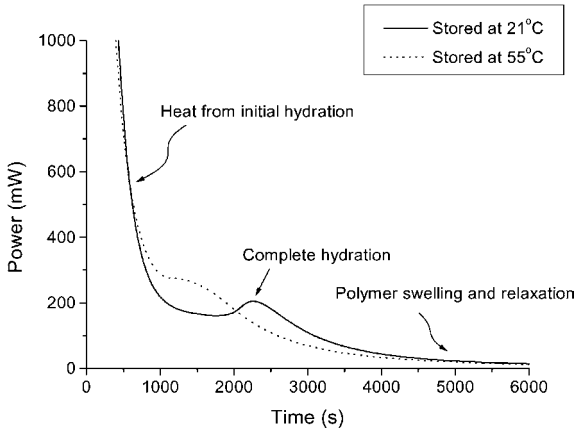

Fig. 4. Power–time data for the swelling of two PEG-based hydrogel segments in water following storage at different temperatures [38].

3.5. Lipids

The study of lipids is of importance pharmaceutically because of their importance in the structure and function of biological membranes. HSDSC allows changes in phase transition properties as a function of temperature and concentration to be investigated for both natural and synthetic lipids [4]. Lipids are usually studied in liposome form, to try to mimic naturally occurring membrane structures; the ability to understand and manipulate membrane function would lead to many applications in pharmaceutical and related areas. HSDSC can be employed simply as a routine analysis tool, to determine the absolute purity of samples, for example, or can be used to investigate physical properties of lipids.

Zhang and Rowe [39] used HSDSC to investigate the phase behaviour of dipalmityl phosphatidylcholine (DPPC) as a function of *n*-butanol concentration. It was observed that the main transition between the P_β and L_α phases was very narrow for the pure lipid but was broadened in the presence of alcohol. (The width of the transition at half-height changed from 0.07°C for the pure substance to 0.37°C in the presence of 30 mg ml^{-1} alcohol.) At higher *n*-butanol concentrations, the lipid became interdigitated prior to melting; the main transition in this case is between the $L_\beta I$ and L_α phases. The main transition temperature was observed to decrease with increasing *n*-butanol concentration. A pre-transition was observed which changed in shape around 10 mg ml^{-1} *n*-butanol, reflecting the transition between the L_β and P_β phases at zero or low concentrations of alcohol or between the L_β and $L_\beta I$ phases at higher alcohol concentrations.

The enthalpies of the transitions were also determined, the $L_\beta I$ to L_α transition having a slightly higher enthalpy than the P_β to L_α transition (reflecting the greater order of the $L_\beta I$ phase compared with the P_β phase). The enthalpy values were observed to differ slightly with different concentrations of *n*-butanol,

presumably reflecting a contribution of the enthalpy of binding of *n*-butanol to each phase.

Further phase changes are known to occur in lipid systems at higher temperatures that involve the formation of three-dimensional, macromolecular structures [40]. Typical examples include the formation of inverted cubic (Q_{II}) and hexagonal (H_{II}) structures. The L_α to Q_{II} phase transition in concentrated solutions of the hydrated form of *N*-methylated dioleoylphosphatidyl-ethanolamine (DOPE-me) has been studied using HSDSC [41]. At high (33 wt.%) concentrations in TES buffer DOPE-me was observed to exhibit different endotherms at different scan rates. At 9°C min^{-1}, two endotherms were observed, a small peak centred at 61.4°C and a larger peak at 66°C. These transitions corresponded with the formation of the Q_{II} and H_{II} phases, respectively. As the scan rate was reduced, the lower temperature endothermic grew in size at the expense of the higher one until, at 1.1°C min^{-1} the endothermic at 66°C had disappeared completely, indicating that the entire sample formed the Q_{II} phase.

3.6. Nucleic acids

In combination with new synthetic methodologies, HSDSC allows investigation of the thermal behaviour of nucleic acids. For instance, Chaires and Sturtevant [42] used HSDSC measurements to characterise the B to Z transition (the B-form of a polynucleotide is right-handed and the Z-form is left-handed; an increase in temperature is often sufficient to convert one form to the other) of the polymer poly(m^5dG-dC). Calorimetric measurements provided an enthalpy change for the transition while the complimentary techniques of circular dichroism (CD) and spectroscopy were used to ascertain the structure of the nucleotide at various temperatures. The HSDSC data showed a B–Z transition centred around 38°C with a second, previously unreported, transition centred at 54°C. The CD data suggested that the higher transition related to a change to an alternate left-handed structure. A helix to coil transition was also noted at approximately 120°C.

Enthalpy values for the transitions were determined from the calorimetric data; ΔH_{cal} values of 0.61 kcal mol^{-1} bp^{-1} for the lower transition and 1.05 kcal mol^{-1} bp^{-1} for the higher transition and ΔH_{vH} values of 68 kcal mol^{-1} for the lower transition and 263 kcal mol^{-1} for the higher transition. (ΔH_{cal} is determined from the area under the curve, ΔH_{vH} is determined using the van't Hoff analysis). The ratio $\Delta H_{vH}/\Delta H_{cal}$ gave the length of the co-operative unit, 110 ± 20 bp in this case. The data suggested that the transition from the B- to the Z-form was enthalpically driven, a conclusion that had been drawn from previous studies.

Nucleotides containing abasic sites (abbreviated to AP, they are produced when a heterocyclic base is removed by the selective hydrolysis of an *N*-glycosidic bond) have also been investigated using HSDSC [43]. The thermal behaviour was studied of a series of DNA duplexes of general structure

Strand 1: CGCATGAGTACGC
Strand 2: GCGTACXCATGCG

where different substituents were placed at position X. Four duplexes were prepared; one contained thymine and hence formed a fully base paired structure (A·T duplex) while the other three contained an abasic site (using a tetrahydrofuran group, A·F, an ethyl group A·E or a propyl group, A·P).

HSDSC measurements showed that the A·T duplex exhibited a transition centred around 75°C, while the three abasic derivatives showed smaller transitions centred at 60°C. The authors suggest that the presence of an abasic site can decrease the stability, transition enthalpy and transition entropy of a duplex structure compared with its Watson–Crick form.

3.7. Proteins

The ability to study the thermal behaviour of proteins is of increasing importance because of their widespread use as therapeutic agents and diagnostic agents but they need careful study because of their propensity to lose both structure and activity when taken out of their natural environment. Proteins are macromolecules and possess secondary and quaternary structures that are stabilised by weak interactions. Many factors can disrupt these forces including pH, ionic strength and temperature. For instance, Riesen and Widmann [44] showed that the thermogram of beef blood haemoglobin recorded at pH 7.06 possesses a clear denaturation endothermic while the same protein recorded at pH 3.32 shows no endothermic signal, indicating that the protein must have denatured

completely before the experiment was conducted. Using HSDSC, it is possible to investigate protein solutions directly. It should be noted that in many cases, protein denaturation is irreversible and that some proteins cannot be investigated using calorimetric methods because they precipitate after denaturing. A common feature observed in protein denaturation thermograms is a difference in heat capacity between the initial and final states of the protein [45], reflecting the fact that the initial and final states of the protein in solution are physically distinct.

It is possible to use calorimetry to study protein interactions in more detail by fitting the data obtained to a suitable model. In simplistic terms, protein denaturation reactions can be classified into two groups; two-state transitions and non-two-state transitions [5]. In the case of two-state ('all or none') schemes there are essentially two macroscopic states, the native state and the denatured state, and the populations of any intermediate states are not significantly populated at equilibrium. For non-two-state reactions some or all of the intermediate states are populated at equilibrium. It has been demonstrated, mostly as a result of calorimetric measurements, that the unfolding of some proteins shows only two-state character, although most proteins exhibit more complex unfolding mechanisms [5]. Two-state models are usually based on the van't Hoff analysis. Finding that the van't Hoff equality holds for a particular set of data gives confidence that the protein denaturation being studied is of two-state character. If the reaction is two state in character then the value of T_m or $T_{1/2}$ should be independent of concentration. An increase in the value of T_m with an increase in concentration suggests a decrease in the degree of oligomerisation, and a decrease in the value of T_m with an increase in concentration suggests an increase in the degree of oligomerisation.

Sturtevant et al. [46] employed HSDSC to study the unfolding of various forms of tropomyosin. Tropomyosin exists in a two chain α-helical coiled coil conformation, and exists in two forms; α,α-tropomyosin and β,β-tropomyosin. The αα-form has a single sulfhydryl group at position 190 while the ββ-form has an additional sulfhydryl at position 36. If the sulfhydryl groups are oxidised then di-sulfide bonds will be made between the two chains; one bond in the αα-form and two in the ββ-form. Some studies have been conducted using forms of tropomyosin which have had the sulfhydryls blocked to prevent chain cross-linking, and it is usually assumed that this does not affect the thermal unfolding of the protein [46]. HSDSC was used to study the oxidised and reduced species of the αα- and ββ-forms and the sulfhydryl blocked (using both carboxymethylation, CM or carboxyamidomethylation, CAM) species of the αα-form. It was shown that for the oxidised and reduced αα-forms, the data fitted a three domain model, while all the data for the other species the data fitted a four domain model. The HSDSC data also showed clearly that blocking the sulfhydryl groups led to an appreciable change in unfolding behaviour.

A more recent application of HSDSC is the study of the thermodynamic effects of protein mutations, an excellent review of which is provided by Sturtevant [47]. In such studies, amino acid changes are made in proteins of known structure and the resultant changes in the thermodynamics of processes, such as unfolding, are determined quantitatively. Stearman et al. [48] studied the protein λ repressor. Three mutant proteins were created. In the first mutant two glycines were replaced with alanines in an attempt to stabilise the α-helix structure (the Ala-46/Ala-48 mutant). In the second mutant, a tyrosine was replaced with a cysteine allowing a di-sulfide bond to form (the Cys-88 mutant) and the third mutant contained both these sets of mutations (the triple mutant). The thermal unfolding of the wild type and mutant proteins was studied using HSDSC since it is possible to obtain information on stability from changes in thermal behaviour; higher transition temperatures suggest the protein is more stable. It was observed that the wild type protein denatured at 53.9°C, the Cys-88 mutant denatured at 62.7°C, the Ala-46/Ala-48 mutant denatured at 62°C and the triple mutant denatured at 70.3°C. The data show that the Cys-88 mutant, possessing one extra di-sulfide bond was more stable than the wild type but less stable than the Ala-46/Ala48 mutant, which possessed a stabilised α-helix structure. The triple mutant was the most stable variant.

3.8. Titration microcalorimetry

The use of titration microcalorimetry allows the introduction of a liquid to a solid or liquid sample. Typically, sample volumes can be up to 3 ml and

titrant volumes up to 10 μl [49]. The titrant is held in an external reservoir and is maintained at the temperature of the sample to minimise thermal shock. The most widespread use of titration microcalorimetry is to measure binding affinities. Typical examples of interactions that may be investigated using titration microcalorimetry include ligand binding, solute–solute interactions, drug–excipient interactions and enzyme–substrate interactions.

The thermodynamics that describe interacting species can be complex. For example, during the transition from one state to another, hydrogen bonds, van der Waals forces and hydrophobic interactions are broken and formed [50]. In the case of free molecules that join to form a complex, solute–solvent interactions are disrupted before complexation can occur. The calorimeter measures the heat changes associated with all these events. It is possible to determine the binding constant, K_b, and the enthalpy of interaction, ΔH_b, for ligand binding in a single titration experiment by fitting the data obtained to a simple kinetic model [51]. Once these data are known it is possible to determine the remaining thermodynamic parameters for the interaction, by application of the relationship given by Eq. (1). This approach presupposes the binding mechanism is known, but most software packages include a range of models and will determine the model that gives the best fit:

$$-RT \ln K_b = \Delta_b G^\circ = \Delta_b H^\circ - T \Delta_b S^\circ \quad (1)$$

Critical micelle concentration (cmc) determinations may be conveniently determined using titration microcalorimetry. Micellisation may be studied using titration calorimetry by injecting a concentrated polymer solution (one that is above its cmc) into a dilute polymer solution (one that is below its cmc) or into water. Following injection, the concentrated polymer solution is diluted and demicellisation occurs. The heat associated with these processes is recorded and plotted versus injection number, polymer concentration or time. The experiment is referenced against a blank experiment whereby water is injected into water such that any heats associated with the injection process itself are removed. When the solution concentration in the ampoule reaches the cmc of the polymer, further injections of concentrated polymer will not result in a demicellisation exotherm. A first-derivative plot of the heat recorded for each injection

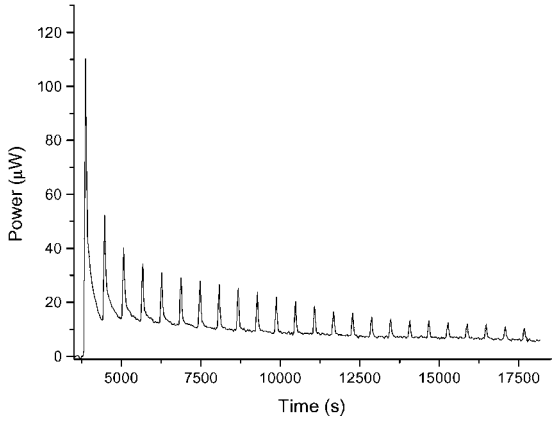

Fig. 5. Power–time curve for the injection of Pluronic F127 (P407) into water using commercial titration apparatus [53].

versus polymer concentration reveals a break point which corresponds with the cmc. Titration microcalorimetry has been used to study micelle formation in, for example, aqueous solutions of SDS [52] and poloxamers [53]. Fig. 5 shows the power versus time data recorded for the injection of concentrated Poloxamer P407 (a block copolymer comprising PEO and PPO) into water [53]. It can be seen that the initial titration produces a large response and the subsequent titrations each produce smaller peaks. The final peaks relate solely to the dilution of the polymer solution and become uniform in size.

An additional benefit of titration microcalorimetry is that one is not limited to the study of aqueous solutions. This is beneficial for the study of, for example, surfactant solutions for which conventional measurements such as surface tension can be almost impossible to make for non-aqueous solvents. The use of non-aqueous suspensions in inhalation aerosols is pharmaceutically important but little is known about how surfactants stabilise these systems. It is difficult to obtain adsorption isotherms for surfactants onto drug particles dispersed in organic solvents because the (usually) high volatility of such solvents can make the measurement of small changes in concentration difficult. Titration microcalorimetry has been used to measure the interactions between two surfactants, oleic acid and span 85, in solutions of salbutamol base in Arcton 113 [54]. It was observed that the interaction of oleic acid with the drug produced an exothermic response while the interaction of span 85

with the drug resulted in an endothermic response. Both surfactants exhibited break points with increasing solution concentration in the presence and absence of salbutamol base. It was concluded that the structure of the surfactant in solution strongly affected the adsorption process.

It is also possible to study the interactions of drugs and excipients with animal cells or bacteria using titration microcalorimetry [49]. In this case, the cells are suspended in a suitable medium or adhered to a surface in the microcalorimetric ampoule and the drug or excipient is introduced by titration. The ampoule may be equipped with an electrode which allows concurrent measurement of pH or oxygen concentration.

4. Summary

Microcalorimetry is ideally suited to the study of physical processes. The inherent sensitivity of the technique means that the heat-flows associated with nearly any physical change can be detected. The nature of the technique means that nearly any sample can be studied — the only prerequisite is that the sample fits within a suitable ampoule (the physical form of the sample being immaterial).

Isothermal microcalorimetry is suited to the study of those systems that are undergoing a change with time autonomously or that can be induced to change by alteration of their local environment (RH, pH, etc.). The technique has been, for instance, used to study a wide range of systems, from relatively simple solution phase reactions to complex heterogeneous pharmaceutical formulations. Examples of solution phase reactions investigated using isothermal microcalorimetry include hydrolysis reactions (ascorbic acid and triacetin, for instance), micellisation of surfactants and enzyme kinetics. Control of the local RH in the calorimetric cell allows the investigation of the recrystallisation properties of amorphous materials and mediates reactions between solid-state materials. Modelling of power–time data allows an estimate to be made of the proportion of a material that will react under certain conditions as well as allowing the determination of a complete range of thermodynamic and kinetic parameters, as long as the reaction mechanism is known.

HSDSC has been used to investigate samples that can be induced to undergo some sort of change with an increase in temperature. Conventional DSC is commonly used for studying physical changes in pure, solid pharmaceuticals (such as glass and other phase transitions) but is not sensitive enough to allow the investigation of solutions or heterogeneous systems. HSDSC, being at least 100-fold more sensitive and allowing the study of dilute solutions, has found many applications with, usually, biological systems. The technique is ideally suited to the study of temperature-driven phase changes in for example solutions of proteins, nucleic acids and lipids. Using the technique in a step-wise isothermal mode also allows rapid screens for drug–excipient compatibility.

Many pharmaceuticals undergo physical changes, either during manufacture or storage, and microcalorimetry has found many applications in this area. Moreover, pharmaceuticals tend to be complex heterogeneous systems that are difficult to investigate using more conventional techniques. Calorimeters measure the heat flows from all the processes that are occurring in a sample simultaneously. This intrinsic property, while having the potential to be the technique's most important advantage, has to date somewhat restricted widespread application of the method. It is to be hoped that recent advances in analysis of calorimetric data, coupled with careful experimental design, will lead to a greater application of microcalorimetry in the pharmaceutical sciences.

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