Review

Microcalorimetry: a response to challenges in modern biotechnology

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Summary

Almost any process in life is accompanied by heat changes which can be monitored by isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC). Both techniques are now established tools in fundamental research but over the last decade a clear tendency towards more problem-driven applications is noted. This review aims at summarizing these problem-oriented applications of microcalorimetry and the solutions both techniques can provide to problems in biotechnology. The biotechnological issues to which microcalorimetry has been successfully applied are as diverse as rational drug design, overcoming drug resistance, optimization of long-term stability of proteins, estimation of the bioavailability of drugs, control of complex pharmaceutical products or the optimization of gene delivery efficiency. The main limitation of microcalorimetry, which is the relatively large amounts of sample necessary for analysis, is less important in the biotechnology sector which frequently uses large-scale produced bulk products for analysis. The recently developed high-throughput DSC and ITC microcalorimeters will additionally reduce the labour intensity of these techniques. Due to the precision of microcalorimetric analyses and the versatility of processes which can be studied, it is expected that ITC and DSC will soon be key technologies in biotechnological research.

Introduction

Challenges to modern biotechnology are multiple. Biotechnological enterprises are more than ever judged by the amount of pharmaceutical products currently in the research and development phase commonly referred to as pipeline. Investors monitor closely the speed by which the development of potential new products advances and evaluate the time which passes between the different phases of clinical trials. In order to obtain a licence of a newly developed product, the requirements brought forward by the national agencies such as the Food and Drug Administration (FDA) have increased enormously over the last decade. Nowadays, more detailed and extensive information on drug efficiency, safety and comparability between different product lots is required to get the permission to commercialize a product. Another factor important for surviving in the harsh biotechnological sector is the better control of the production processes of existing products to reduce the percentage of products which do not pass final quality control and which cannot be put onto the market.

Over the last three decades microcalorimetry has established its place as a key technique in the fundamental research on biomolecules. More recently, however, the use of microcalorimetry has become more applied and problem-driven. In general, the research and development processes of a pharmaceutical product represent always a combination of empirical and rational approaches. There are now a considerable number of examples which illustrate that the use of microcalorimetric techniques can render research and development more rational and less empirical. This is exemplified by the optimization of lead drug molecules which is traditionally a rather random-based process. However, the knowledge of the enthalpic and entropic contributions to lead molecule binding at the drug target, as determined by isothermal titration calorimetry (ITC), offers some clues on how to rationally optimize this compound. Protein stability is another parameter which frequently hampers the development of a new product. Protein stability can be improved by the addition of excipients or protein engineering. The identification of the optimal excipient is generally achieved by time-consuming screening processes. However, the stabilizing effect of a given excipient on a protein can be determined by differential scanning calorimetry (DSC) in less than an hour. Such data in turn allow...
narrowing down the number of excipients which are to be submitted to stability tests. In analogy, the consequences of protein engineering on the stability of a protein are rapidly and precisely assessed by DSC. There is no doubt that rendering research more rational will shorten the ‘time to market’ of products, a key parameter in the biotechnology sector. This review aims at summarizing the scope of microcalorimetric techniques in applied and problem-driven research.

**Isothermal titration calorimetry**

There are two main modes in microcalorimetry, namely ITC and DSC. In general, binding of a ligand to a macromolecule will either generate or consume heat which is measured in an ITC experiment. A molecular interaction between two ligands can be defined by the following equation which forms the basis for an ITC analysis:

$$\Delta G = -RT \ln K_D = \Delta H - T \Delta S$$

The first part of this equation implies that the change in the Gibbs free energy ($\Delta G$) correlates with association constant $K_D$ because $R$, the gas constant, and $T$, the absolute temperature, are constant. The dissociation constant $K_a$, which is commonly used to quantify the affinity between two ligands, is the inverse of $K_D$. The second part of this equation illustrates that the sum of enthalpy ($\Delta H$) and entropy ($\Delta S$) changes define the free energy ($\Delta G$) and consequently the affinity of an interaction. A major advantage of ITC experiments is the fact that $K_D$ and $\Delta H$ are measured in a single experiment. Having measured these two parameters the remaining variables $\Delta G$ and $\Delta S$ can be derived.

The experimental set-up of an ITC experiment is as follows. The macromolecule is placed into the sample cell of the calorimeter and is titrated at constant temperature with the ligand in a way that saturation of the macromolecule in the sample cell occurs. During this titration heat changes are constantly monitored which is illustrated in the top panel of Fig. 1 which shows the titration of the TbpA subunit of the transferrin receptor of *Neisseria meningitidis* with apo- and holo-transferrin (Krell et al., 2003). Each peak represents the heat changes caused by the injection of one aliquot of transferrin into TbpA. Subsequently these raw data are integrated, normalized using the concentration of both ligands, corrected for dilution events and submitted to curve fitting using interaction models (Fig. 1, bottom). From this fit, two parameters are directly obtained which are the binding constant $K_a$, which is related to the slope at the midpoint of saturation (the steeper the tighter is binding) and the enthalpy change $\Delta H$ which for high-affinity binding corresponds roughly to the value where the curve meets the y-axis. As stated above the $\Delta G$ and $\Delta S$ values are then calculated using the above equation.

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Isothermal titration calorimetry data for the titration of the TbpA subunit of the transferrin receptor of *Neisseria meningitidis* with iron-loaded (A) or iron-free (B) human transferrin. Top: raw titration data. For clarity, curves have been off-set on the y-axis. Bottom: integrated and normalized raw data fitted with the ‘One binding site model’ of ORIGIN (Microcal, Amherst, Massachusetts). Thermodynamic parameters derived are: (A) $K_D = 69$ nM, $\Delta H = -28.6$ kcal mol$^{-1}$; (B) $K_D = 3.7$ nM, $\Delta H = -41.8$ kcal mol$^{-1}$. Figure reprinted from Krell and colleagues (2003): *J Biol Chem* **278**: 14712–14722, with permission from American Society of Biochemistry and Molecular Biology (ASBMB).

As a result of an ITC experiment four parameters ($\Delta G$, $\Delta H$, $\Delta S$ and $K_D$) are obtained. The information obtained from an ITC experiment is thus not solely on the strength of interaction ($K_D$) but also on the forces that drive this interaction ($\Delta H$, $\Delta S$). Other alternative techniques such as fluorescence quenching or equilibrium dialysis provide primarily information on the binding affinity. Our general understanding of the structural features that determine the entropic or enthalpic nature of interaction between two ligands is growing. In this context the advantage of determining these parameters by ITC has proven to be increasingly useful in tackling different biotechnological challenges which is illustrated in this review.

**Identification and optimization of lead compounds in drug development**

Isothermal titration calorimetry plays a role in the identification but more importantly in the optimization of lead compounds. Lead compounds are typically defined by an
affinity for their target of below 10 μM. The use of ITC in the identification of inhibitors is illustrated on the bacterial two-component regulator systems (TCS) which are frequently found essential for the virulence of a pathogen and are therefore attractive targets for the development of inhibitors (Stephenson and Hoch, 2004). Previous attempts to develop specific inhibitors against the sensor kinase component of TCSs were crowned by limited success. Compounds identified were shown to have secondary effects which appear to be due to the fact that the majority of them interfere with nucleotide-binding domain (Stephenson et al., 2000) which increases the risk of an interaction of compounds with other ATP-utilizing proteins.

Gene expression studies in vivo have identified a number a small signal molecules, e.g. toluene, which activate the TodS/TodT TCS (Lacal et al., 2006). Isothermal titration calorimetry studies showed that these molecules bound with sub-micromolar affinity to the sensor kinase TodS thereby stimulating its kinase activity (Busch et al., 2007). Surprisingly, further ITC experiments revealed that a number of small molecules, such as o-xylene, which did not activate the system in vivo also bound with high affinity to TodS at the same binding site as the one used by toluene (Busch et al., 2007). These compounds were termed antagonists and additional studies revealed that these antagonists inhibit the agonist-mediated upregulation of the kinase activity. These studies thus demonstrate that the assessment of the inhibitory activity of signal antagonists might be a potentially fruitful alternative to develop specific inhibitors of TCS.

A critical step in the development of a drug is the optimization of lead compounds as affinities in the lower nanomolar or sub-nanomolar range are necessary for a clinical application of the drug. This lead optimization is a major bottleneck in drug development (Ruben et al., 2006) and many drug development projects fail to jump this hurdle. However, the pioneering work of the group of Ernesto Freire has provided some clues on how to optimize lead compounds in a more rational way using ITC.

The binding affinity is determined by the Gibbs free energy change $\Delta G$, which is the sum of changes in enthalpy and entropy $\Delta G = \Delta H - T\Delta S$. To increase an affinity, $\Delta G$ has to be made more negative. This can be achieved in several ways: either by making $\Delta H$ more negative (enthalpic optimization) or by making $\Delta S$ more positive (entropic optimization) or by an appropriate combination of both. Our understanding is advancing on the structural determinants of an inhibitor which impact on its binding entropy and enthalpy to a macromolecule. This in turn offers the possibility to propose structural changes to an inhibitor to rationally optimize one or the other parameter.

The entropy term is primarily composed of the solvation entropy, associated with the burial of non-polar groups from the solvent, and the conformational entropy which is a function of the loss of conformational degrees of freedom upon binding. A structural modification, for instance, which renders the inhibitor more hydrophobic, increases the solvation entropy. This hydrophobicity-based optimization has certainly its limits as the drug has to be soluble under physiological conditions. Further contributions to the entropy term can be associated with the burial of polar surfaces and the liberation of water, which is discussed in more detail in Ladbury (1996).

Enthalpic contributions reflect the strength of the inhibitor interactions with the protein (H-bonds, van der Waals interactions etc.) relative to those with the solvent. The optimization of the enthalpy term involves the optimization of binding distances and angles of inhibitor–protein bonds to achieve an optimal key-to-lock fit. This optimization is based on high-resolution structural data of protein/ligand complexes. Further reading on enthalpy–entropy optimization can be found in Velázquez-Campoy and colleagues (2000), Holdgate (2001) and Ruben and colleagues (2006).

The Gibbs free energy change $\Delta G$ is the sum of $\Delta H$ and $\Delta S$ and an infinite combination of different $\Delta H$ and $\Delta S$ values can add up to a given $\Delta G$ value. In the case of the inhibitors of the proteases from HIV (Velázquez-Campoy et al., 2001) and Plasmodium falciparum (Nezami et al., 2003; Ruben et al., 2006) it has been shown that low-affinity inhibitor binding can be achieved using a wide range of $\Delta H/\Delta S$ combinations including frequently unfavourable contributions of enthalpy (positive value) and entropy (negative value). As the affinity increases this range of $\Delta H/\Delta S$ narrows down. Most importantly, however, was the observation that very high-affinity binding is only achieved when both, enthalpy and entropy changes, are favourable (Ruben et al., 2006).

This is important information for lead optimization which is here illustrated by a study carried out by Pfizer on the optimization of renin inhibitors (Sarver et al., 2007). Using high-throughput screening a lead compound was identified which bound with a $K_D$ of 3.57 μM to its target renin. This binding reaction was driven by favourable enthalpy changes ($\Delta H = -9.27 \text{ kcal mol}^{-1}$) but counterbalanced by unfavourable entropy changes ($T\Delta S = -2.00 \text{ kcal mol}^{-1}$). Efforts were thus made to optimize the entropy term which was achieved by designing a compound which had a favourable entropy term ($T\Delta S = 0.43 \text{ kcal mol}^{-1}$) and an almost unchanged enthalpy change. As a result the affinity of the optimized inhibitor was 45-fold increased as compared with the initial lead compound.

Therefore, the potential of ITC to dissect $\Delta G$ into $\Delta H$ and $\Delta S$ combined with the knowledge gained from several different systems indicating that both $\Delta H$ and $\Delta S$ have to be favourable for high-affinity binding provides the basis for a more rational optimization of lead compounds.
Estimating the bioavailability of drugs

The clinical efficiency of a drug is determined by many factors. One of them is the affinity for its target, but another important factor is the ability of drugs to bind non-specifically and reversibly to serum proteins, thereby reducing the concentration of free, bioavailable drug. The bioavailability of HIV protease inhibitors was assessed by microcalorimetric titrations of the serum proteins human serum albumin (HSA) and α1-acid glycoprotein (AAG) with inhibitors already in clinical use or in development (Schön et al., 2003). Both proteins are known to bind drugs in a non-specific way, thereby lowering their effective concentrations. Under conditions simulating in vivo concentrations the different inhibitors bound to AAG with affinities ranging from 0.77 to 27 μM (Schön et al., 2003). Based on these data and the affinities for the binding of the inhibitors to the HIV protease, the drug concentration necessary to inhibit the proteases in vivo was estimated. To achieve the inhibition of 95% of the protease an almost 12 times higher concentration of the inhibitor nelfinavir is required as compared with calculations which include the absence of serum proteins. For another inhibitor, KNI-764, this ratio is much more favourable as the inhibitor concentration has to be elevated only by a factor 2.3 to achieve the protease inhibition one would have observed in the absence of serum proteins. Apart from the affinity of the drug for its target, information on the bioavailability can be easily obtained by ITC which represents an additional important criterion for drug development.

Overcoming drug resistance in a more rational way

The development of clinically applicable inhibitors of the HIV protease was a major breakthrough in fighting this virus. However, the antiretroviral therapies are hampered by the emergence of drug resistance. This resistance is mainly caused by mutations in the HIV protease and drug-treated patients were shown to have a significantly higher amino acid polymorphism of the HIV protease than untreated patients (Ohtaka et al., 2004). This polymorphism is observed in at least 49 amino acids of the 99 residues of each protease monomer (Ohtaka et al., 2004). Some mutations were shown to lower the affinity of inhibitors for its target by a factor of 1000 (Velázquez-Campoy et al., 2003). A screening of compounds had thus to be undertaken to identify novel inhibitors which bind with high affinity to native as well as mutant protease. The group of E. Freire has extensively studied the thermodynamics of the binding of inhibitors, either in clinical use or in development, to native and mutant proteases. Data were reported in some 20 publications which are summarized in Ohtaka and Freire (2005). The authors noted that some inhibitors could cope better than others with different mutations. The obvious question concerned thus the properties which enable some inhibitors to cope better with mutations than others. In trying to respond to this question the individual determination of the entropic and enthalpic contribution to binding, as determined by ITC, was of enormous help.

This is exemplified by binding studies of the inhibitors amprenavir and TMC-126 to the native protease as well as to mutants I50V and V82F/I84V (Ohtaka et al., 2002). All mutations are located in the protease-active site. The mutations I50V and V82F/I84V lower the binding affinity of amprenavir by a factor of 147 and 104 respectively. However, the same mutations lower the affinity of inhibitor TMC-126 only by a factor of 16 and 11 respectively. The binding of both inhibitors to both mutants was more favourable as compared with the wild-type protein, whereas the entropy changes remained almost unchanged for the binding of amprenavir. The capacity of TMC-126 to cope better with the mutation is thus due to the fact that the loss in binding interaction (more unfavourable enthalpy) is compensated by a gain in entropy. This gain is due to an increased conformational flexibility within the protein–inhibitor complex. This allows that the inhibitor TMC-126 adapts better to the new shape of the binding pocket. This gain in entropy does not occur in amprenavir, hence a lower affinity for both mutants.

Based on these results a strategy was established for the design of inhibitors against heterogeneous targets, such as the totality of HIV protease variants (Ohtaka and Freire, 2005). These inhibitors are referred to as adaptive inhibitors. The initial part of this strategy is to map the sequence polymorphism of a drug target onto its three-dimensional structure in order to identify the invariable amino acids in the substrate/inhibitor binding site. It is important to note that the drug-induced amino acid polymorphism in the HIV protease has not occurred in random fashion, as mutant proteins have to maintain sufficient catalytic activity to guarantee viral replication, but are insensitive to inhibitors. Therefore, conserved regions exist of which mutation would result in a loss in the catalytic activity of the enzyme. The drug molecule is conceived in a way that it establishes its strongest interactions with those conserved regions of the binding pocket. The variable regions of the binding site are covered by flexible elements of the drug molecule which permits an optimal accommodation of the drug molecule to different mutant proteases. This new strategy is of general relevance in the field of drug research.

Exploiting the information on binding stoichiometry

The examples cited so far illustrate the interpretation of the parameters of binding. However, another parameter,
the binding stoichiometry, can also be determined from an ITC experiment. This information can be used to determine the percentage of 'active macromolecule' in a given sample which serves to verify, for example, that protein purification has not caused protein inactivation or denaturation. This application is here illustrated on the development of a vaccine against meningitis caused by serogroup B \( N. meningitidis \), against which currently no vaccine exists. The two subunits of the transferrin receptor of \( N. meningitidis \), TbpA and TbpB, are candidates for such vaccine (Rokbi \textit{et al.}, 2000; West \textit{et al.}, 2001). The purification of both proteins involves their extraction from the membrane using detergents. The titration of purified TbpA with human holo- and apo-transferrin is shown in Fig. 1. The point of inflection of both sigmoid curves corresponds to the stoichiometry which can be read from the lower x-axis representing the transferrin–TbpA ratio. In both cases the point of inflection is at a molar ratio close to 0.5. As we know that one molecule of transferrin binds to a TbpA dimer, the TbpA sample analysed corresponds to a protein preparation containing only active TbpA as measured by their capacity to bind transferrin. If saturation had occurred at a lower ratio, let's say 0.3, the amount of active protein would have been only 60%. Figure 1 also illustrates that the stoichiometry is determined more precisely for higher-affinity binding (the steep slope observed for apo-transferrin in Fig. 1) than for lower-affinity binding events (the less steep slope for the binding of holo-transferrin). The ITC-based evaluation of the active protein fraction has also been of help to identify the optimal parameters for the purification of the TbpB subunit of the receptor (Krell \textit{et al.}, 2002).

It can be concluded that ITC is a very suitable tool for the control of pharmaceutical products as it determines independently the binding parameters and the fraction of active product which gives thus a very clear picture of the product analysed. Using other alternative techniques such as ELISA, fluorescence quenching measurements or BIACore this dissection is either impossible or subject to a considerable error.

**ITC to study enzyme kinetics**

The applications of ITC so far reported are all on the study of ligand binding. However, ITC can also be used to determine the kinetic constants of enzymes, which implies that not heats of binding but heats resulting from catalysis are monitored. The most frequently used method consists in a single injection of substrate into enzyme (Todd and Gomez, 2001). Heat changes are monitored until all the substrate injected is converted into product. From such data the \( K_m \), \( k_{cat} \) and molar enthalpy of the enzymatic reaction can be determined. If the experiment is repeated in the presence of an inhibitor the inhibition constant (\( K_i \)) of an inhibitor can be obtained.

Todd and Gomez (2001) have used this method to determine the kinetic parameters for representative examples belonging to the six classes of enzymes (EC1–EC6). The authors conclude that the kinetic parameters determined by microcalorimetry are close to the values reported in the literature, determined by standard assays.

Microcalorimetry-based enzyme kinetics is a suitable alternative for cases where standard assays can not be applied which is here exemplified by the analysis of inhibitors of orotidine monophosphate decarboxylase (Poduch \textit{et al.}, 2006). Spectrophotometric assays are not suitable for inhibitor studies of this enzyme as substrate, product and inhibitors have overlapping spectral absorption properties. Furthermore, a HPLC-based enzyme assay was found to be unsuitable for inhibitor studies. In their search for an alternative the authors found ITC a very satisfactory alternative. Advantages of microcalorimetry-based enzyme kinetics over standard techniques include the possibility to study enzymatic reactions under conditions such as poor transparency of the reaction solution and there is furthermore no need to use chromogenic, fluorogenic or radioisotope-labelled ligands. Further examples of microcalorimetry-based enzymology are found in Bianconi (2003) and Spencer and Raffa (2004).

**Differential scanning calorimetry**

The second principal microcalorimetric technique is differential scanning calorimetry or DSC. Using this mode a temperature gradient is applied to a sample and heat changes associated with the thermal unstructuring are monitored. For proteins, denaturation processes give rise to peaks in DSC thermograms, indicative of an endothermic event. This endothermic nature of protein unfolding is due to the fact that denatured protein has higher energy than the native form. For further reading on DSC please refer to Freire and colleagues (1990) and Ibarra-Molero and Sanchez-Ruiz (2006).

The thermal denaturation of hexokinase A is illustrated in the bottom trace of Fig. 2 (Tiwari and Bhat, 2006). This monomeric protein was shown to consist of two structural domains (Bennett and Steitz, 1978). The DSC analysis of this protein shows two partially overlapping peaks (Fig. 2, bottom trace) which were previously attributed to the consecutive and almost independent denaturation of the two structural domains (Catanzano \textit{et al.}, 1997). The obtained curve can be deconvuluted into two separate events. For each event two principal parameters can be obtained. The first parameter is the midpoint of protein unfolding transition or \( T_m \), corresponding to the temperature at the peak summit. At this temperature half of a given entity is present in its natural state, the other half in its denatured state.

\[ Q = \frac{dH}{dT} \]

Where \( Q \) is the heat flow, \( dH \) is the enthalpy change and \( dT \) is the temperature change. The integral of the heat flow is the cumulative heat flow, which is then used to determine the calorimetrically obtained melting curve. This curve can be fitted to a single or double exponential function, allowing the calculation of the half-life or the number of transitions.

**References**

Krell \textit{et al.}, 2002; Todd and Gomez, 2001; West \textit{et al.}, 2001; Poduch \textit{et al.}, 2006; Freire and colleagues (1990); Ibarra-Molero and Sanchez-Ruiz (2006); Bianconi (2003); Spencer and Raffa (2004).
The second parameter is the enthalpy change ($\Delta H$) which is represented by the peak areas and is an indication of the energy necessary to denature a given entity. The use of DSC in problem-driven research is illustrated below.

Towards a rational identification of conditions for optimal protein stability

Prior to clinical studies data need to be presented that demonstrate that a given pharmaceutical product is stable over a significant period of time. The failure to generate such data can result in a significant delay in the development process. To address this question, stability tests are conducted in which different properties of a pharmaceutical product stored under different conditions are monitored over a time span, e.g. 1 year. Storage conditions can vary, for example, in the pH, ionic strength, temperature, preservatives or stabilizing agents (for further reading see Chi et al., 2003). The optimization of the stability of a pharmaceutical product was traditionally based on trial-and-error-based screening. However, DSC was shown to provide a more rational basis for the selection of storage conditions.

Remmle and colleagues (1998) investigated solution conditions for the stabilization of the interleukin-1 receptor. The formulation of this pharmaceutical protein required the presence of a preservative, which is necessary for multidose products. The authors analysed by DSC the protein in the presence of various preservatives. In parallel a 2-month stability test of the protein in the presence of different preservatives was conducted in which the percentage of native protein was monitored. There was a direct correlation between the thermostability determined by DSC and the amount of native protein seen in the stability test. This correlation between thermostability determined by DSC and long-term stability can thus be exploited to rationally and rapidly identify promising conditions. In initial DSC experiments the pH optimum for protein stability is determined, which is followed by excipient screening at the pH optimum. For example, the excipient screening reported by Tiwari and Bhat (2006) has shown that sorbitol is the most potent additive. The addition of 1.5 M sorbitol increased the $T_m$ of the two events seen in the DSC analysis by 13 and 7 degrees (Fig. 2).

Assessment and optimization of the intrinsic stability of proteins

Apart from the identification of optimal conditions which stabilize a product, the intrinsic stability of a therapeutic protein is another crucial issue, which can also be optimized. Lacking intrinsic stability of a protein can affect protein expression and consequently protein misfolding reduces the fraction of active protein in a protein sample (Martsev et al., 1998). A DSC study of 17 human or humanized therapeutic antibodies was reported by Garber and Demarest (2007). The $T_m$ values for the antigen-binding fragments (Fabs) were found to vary largely from 57 to 82°C. Interestingly, antibodies with low thermal stabilities were found to aggregate and to express poorly. A sequence analysis of the antibodies studied permitted the identification of amino acids which were responsible for the reduced thermal stability. This study provides thus a guideline for stability optimization of other diagnostic and therapeutic antibodies by protein-engineering techniques.

Differential scanning calorimetry analyses are also being used to precisely monitor the increase in thermal stability caused by site-directed mutagenesis, which permits a precise quantification of the stabilizing or destabilizing effect of each amino acid replacement. This is illustrated here on the stability optimization of the antitetanus toxoid human Fab fragment (Demarest et al., 2006). The expression of this protein in *Escherichia coli* resulted only in a very low yield of functional protein and it was hypothesized that this was caused by a lacking intrinsic stability. Forty-five amino acids of this Fab were submitted to saturation mutagenesis. Using automated screening the approximate thermostabilities of some 4000 library members were determined. Fabs containing several mutations which increased the apparent thermostability were generated as purified recombinant proteins and...
analysed by DSC. The identified mutants with increased thermal stabilities were shown to express with higher functional yields in *E. coli*.

**Control of complex biological products**

The vaccine against the influenza virus is a very complex pharmaceutical product. The vaccine is fabricated from three different virus strains which are representative members of the subtypes H1N1, H2N2 (belonging to type A viruses) and of type B. The production of this vaccine includes the culture of these three virus strains, virus purification, which is followed by an inactivation procedure. Figure 3 shows the DSC thermograms of three purified and active virus samples belonging to the three subtypes mentioned above (Krell et al., 2005). These strains were used to produce the influenza vaccine during the years 2000–2004. The thermograms of the different virus strains are entirely different showing two to four major thermal transitions. Furthermore, the analysis of two other viruses belonging to subtypes H1N1 and H3N2 by Epand and Epand (2002) using the exact experimental conditions as in Krell and colleagues (2005) revealed that even virus strains belonging to the same subtype have entirely different thermograms. Very closely related viruses which share a high degree of sequence identity have thus very different denaturation characteristics. The denaturation profile is thus very specific for a given virus strain which is a property which could be exploited for in-process control purposes. Krell and colleagues (2005) have shown that the DSC analysis is also of interest in monitoring the subsequent steps in vaccine preparation such as the virus inactivation using β-propiolactone.

**Impact of freezing and drying on pharmaceutical products**

Many storage conditions of biopharmaceuticals involve freezing or drying. Both processes cause different types of stress to the protein leading often to protein denaturation which typically manifests as aggregation. Traditionally, the identification of optimal freezing or drying conditions was an empirical trial-and-error approach. Modern biophysical techniques, including DSC, permit a more rational identification of such conditions, primarily by getting insight into the molecular mechanisms which underlay protein aggregation.

Apart from measuring the thermal denaturation of biomolecules, DSC can also be used to determine the glass transition temperature or *Tg*′ of a buffer component. *Tg*′ is the temperature below which the physical properties of amorphous materials vary in a manner similar to those of a crystalline phase (glassy state), and above which amorphous materials behave like liquids. Information on *Tg*′ is a powerful means to monitor processes which occur during freezing of biopharmaceuticals. This is illustrated here by work aimed at optimizing the long-term stability of frozen samples of an Fc (constant part of an antibody) fusion protein (Piedmonte et al., 2007). The authors were facing the problem that this protein denatures and aggregates over time when stored at −30°C. The protein has been formulated with 5% (w/v) sorbitol which is a frequently used protein stabilizer. Over a period of 20 weeks samples of frozen protein were analysed by DSC. Thermograms show that two *Tg*′ transitions at −20°C and −8°C appeared over time which can be attributed to crystalline sorbitol. In other words, the stabilizing agent sorbitol crystallizes over time when stored at −30°C. During freezing, however, the stabilizing agent must remain in the same phase as the protein to ensure protein stability. By crystallizing, sorbitol is phase-separated from the protein leading to its aggregation.

The crystallization of protein-stabilizing agents during freeze-drying has also been shown to reduce protein stability. Krielgaard and colleagues (1999) demonstrate for a lipase that mannitol crystallization during freeze-drying affords less stabilization relative to that seen in the absence of additives. Subsequent DSC studies demonstrated that the addition of sucrose or NaCl inhibits mannitol crystallization during freezing (Hawe and Frieß, 2006).

**Optimization of non-viral gene delivery**

Enormous hopes rest on the use of gene therapy or DNA-based vaccines for the cure and prevention of...
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Whole-cell analysis of bacteria

The examples so far cited on the use of DSC involve the analyses of purified macromolecules and viruses. However, during the last two decades we have seen a continuous output of literature on the DSC analysis of whole bacteria. As expected, the heat denaturation profiles of whole bacteria are complex and consist of multiple peaks, e.g. nine, in the case of Campylobacter (Nguyen et al., 2006). Based on the data gathered over the last two decades it is now possible to attribute some peaks to the unfolding of certain cellular components such as lipids, ribosomes and DNA (Mackey et al., 1991; Nguyen et al., 2006). For several microorganisms the comparison of DSC data with cell survival assays revealed that cell death coincides with the unfolding of ribosomes (Anderson et al., 1991; Nguyen et al., 2006). The major application of such studies is aimed at understanding and improving the heat inactivation of bacteria and bacterial spores which is of importance in guaranteeing food safety (Nguyen et al., 2006; Stecchini et al., 2006). Furthermore, DSC experiments at low temperature have been reported to study the stabilization of frozen bacteria, e.g. lactic bacteria (Fonseca et al., 2006), which play a major role in food industry.

Other applications

This review does not pretend to be a complete summary of biotechnological applications of microcalorimetric techniques. A few other applications are briefly mentioned here to underline the versatility of this technique in problem-motivated research. The determination of the solubility of a drug in a solid or semi-solid matrix is not a straightforward task as approaches to determine solubility of compounds in liquid are not applicable. However, information on the amount of dispersed and dissolved drug in a solid delivery system is necessary to predict the release characteristics of a drug. Differential scanning calorimetry-based techniques have been reported which have successfully addressed this problem (Gramaglia et al., 2005; Oladiran and Batchelor, 2007).

The activity of many biomolecules and drugs crucially depends on whether they bind to biological membranes and whether they translocate to the opposite lipid leaflet and the trans aqueous compartment. An ITC-based method has been described which quantifies the membrane binding and release of drugs (Tsamaloukas et al., 2007). Experiments consist basically in titrations of lipid vesicles into a drug solution to study binding and, alternatively, the titration of drug-loaded lipid vesicles into buffer to quantify the drug release.

Differential scanning calorimetry has found extensive use in the optimization of controlled drug delivery techniques. Among the different approaches in this field is the encapsulation of a drug in different biopolymers. Some of these polymers are thermostable, which implies that they swell in function of the temperature, regulating thereby the rate of drug release. Differential scanning calorimetry has played a central role in developing these
new materials and to characterize the release process (Wu et al., 2005).

Conclusions

What are thus the advantages which make microcalorimetry to a technique of enormous value in biotechnology? Heat changes accompany almost any process in life and microcalorimetry permits to detect them directly. Two major advantages arise from this fact. First, as heat changes are a direct consequence of a process there is no need to alter artificially the participants of a given process, for instance, by labelling. Second, as heat changes are almost ubiquitous the range of different processes which can be studied is enormous and this review aims at giving a glimpse of it. This diversity implies that answers to questions which are very different in type can be obtained.

Another advantage of microcalorimetry which makes it suitable for biotechnological exploitation is its precision and the reproducibility. The error associated with the binding constant determined by ITC is typically in the range of 5% and the error associated with $T_m$ determined by DSC is around 0.1°C. This high precision and reproducibility make microcalorimetry an ideal candidate for the replacement of traditional, less precise quality control procedures for pharmaceutical products.

Traditionally, microcalorimetric analyses are known to be labour intensive as samples had to be loaded manually into the microcalorimeter, making it little suitable for a biotechnological use which frequently requires high-throughput and little labour intensive approaches. With the development of AutoITC and Capillary DSC by Microcal (http://www.microcal.com) first steps have also been made to make microcalorimetry less labour intensive. These instruments allow the conduct of 100 ITC experiments per week or 50 DSC scans per 24 h in an almost autonomous fashion. In addition, experiments can be carried out in almost any solvent.

The requirement of relatively large amounts of sample is commonly given as the primary limitation of microcalorimetry. However, biotechnological research is often supported by a medium- to large-scale production of given product and in this context this limitation appears to be much less important. Taken together it seems therefore likely that microcalorimetry will soon count among the key technologies in biotechnological research.

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