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Clinical application of plasma thermograms. Utility, practical approaches and considerations

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ABSTRACT

Differential scanning calorimetry (DSC) studies of blood plasma are part of an emerging area of the clinical application of DSC to biofluid analysis. DSC analysis of plasma from healthy individuals and patients with various diseases has revealed changes in the thermal profiles of the major plasma proteins associated with the clinical status of the patient. The sensitivity of DSC to the concentration of proteins, their interactions with other proteins or ligands, or their covalent modification underlies the potential utility of DSC analysis. A growing body of literature has demonstrated the versatility and performance of clinical DSC analysis across a range of biofluids and in a number of disease settings. The principles, practice and challenges of DSC analysis of plasma are described in this article.

1. Introduction

Differential scanning calorimetry (DSC) is an established, highly sensitive thermal analysis technique. DSC has been broadly applied in the life sciences to measure the heat profiles of biomolecules with application to areas including protein engineering, biopharmaceutical formulation and the study of various biomolecular interactions such as protein–ligand and protein–protein interactions [1]. DSC normally uses highly purified biomacromolecules to provide detailed and precise thermodynamic information about their stability or reactivity. We describe here a less conventional application of DSC to the analysis of complex mixtures. DSC is sensitive to the extensive properties of a solution of macromolecules and therefore is useful for monitoring concentrations of components in mixtures. This makes possible the application of DSC to provide a signature denaturation profile (thermogram) of a complex mixture based on the thermal stability of its individual components. The DSC thermogram of a mixture provides a unique signature analogous to a spectrum of a multicomponent mixture, or to a chromatographic elution profile, but one that is based on a unique underlying physical property, thermal stability. The goal of DSC studies in such a case is not detailed thermodynamic analysis but rather a more qualitative profiling of complex samples. Thermograms of mixtures do retain quantitative features that may be used for statistical comparisons of samples. Recently DSC has been applied in this novel direction to the analysis of clinical biofluids. Table 1 shows publications in this emerging area of DSC analysis. The majority of the publications address the analysis of human blood plasma or serum with other studies exploring the use of cerebrospinal fluid and, most recently, brain tumors. The clinical areas of investigation are diverse encompassing a range of cancers, autoimmune diseases, infectious disease, chronic health conditions, healthy subjects, athletes and disease controls. These studies have shown that DSC can detect differences in thermograms of biological samples associated with health status. This presents the potential of DSC to complement existing clinical diagnostic approaches.

DSC has a number of attributes that underlie its potential utility as a new diagnostic technology. DSC detects with high sensitivity small heat changes associated with thermally-induced events. It is a universal detector which does not rely on any form of labeling or derivatization of analytes. DSC also does not require any pre-analytical fractionation allowing the analysis of complex mixtures such as blood plasma. As was mentioned, DSC thermograms reflect the extensive properties of a sample and will therefore correspond to changes in the concentration of component proteins associated

Abbreviations: DSC, differential scanning calorimetry; T_m, melting temperature; ΔH, denaturation enthalpy; T_{max}, temperature of the peak maximum; T_{FM}, first moment temperature; C_p^{ex}, excess specific heat capacity; HSA, human serum albumin; IgG, immunoglobulin G.

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with clinical status. DSC is sensitive to any changes in the thermal stability of analytes. In the case of the plasma proteome, structural modifications to plasma proteins or changes in intermolecular interactions associated with a disease state will be sensitively detected by DSC. Changes in thermograms associated with disease conditions are typically manifested as temperature-shifted profiles. One hypothesis for disease-altered thermograms are that these could reflect the "interactome" concept [2]. This concept describes plasma as being composed of a network of protein–protein, peptide–protein and metabolite–protein interactions which one could envisage could be affected by the presence of disease biomarkers. These altered interaction networks would affect the denaturation properties of plasma and result in changes in DSC thermograms. DSC analysis of clinical samples has shown that, relative to control populations, thermograms are altered in shape and shifted to higher temperatures in a number of diseases. Further, thermograms have been shown to be sensitive to disease burden [3] as well as to patient therapy [4]. The growing body of literature applying DSC to different biological samples in an ever increasing number of diseases presents the exciting possibility that DSC might find a place as a useful clinical tool. The aim of this article is to outline the principles and practice of plasma thermogram analysis and to discuss the development of the approach in moving from basic research investigations to the clinical arena.

2. Principle of plasma thermograms

DSC is the method of choice for thermodynamic studies of protein denaturation, where temperature-induced unfolding of proteins can be directly measured. DSC monitors heat changes associated with the thermal denaturation of biomolecules providing a direct quantitative measurement of the thermostability of protein(s) in terms of a denaturation curve (or thermogram). DSC profiles (or thermograms) are unique for each biomolecule as a consequence of unique structural motifs and molecular forces. Under a given set of buffer conditions, every protein has a thermogram with a characteristic melting temperature (T_m) and denaturation enthalpy (ΔH) that provides a fundamental thermodynamic signature for that protein. DSC is typically applied to the analysis of a single purified biomolecule or a biomolecule with an interacting partner. Recently, the utility of DSC for the analysis of complex protein solutions has been explored in the area of clinical diagnostics. DSC thermograms are directly related to the mass of proteins present. For example, if the weight concentration of a protein is doubled, the calorimetric heat response will also double. Likewise, in a solution containing a mixture of proteins, the relative heat response will correspond to the total mass of proteins present. Fundamentally, this property constitutes the basis for DSC-based diagnostic applications since thermograms of protein mixtures can be deconvoluted into characteristic denaturation curves of individual protein components. In a non-interacting mixture, each protein will have a characteristic curve shape, T_m and ΔH. Thus, the thermogram observed for the mixture can be represented as the sum of all constituent individual protein thermograms weighted according to their relative molar mass and concentration. We have shown this to be the case for the plasma thermogram obtained from healthy subjects [5].

A normal thermogram and its deconvolution are shown in Fig. 1. The normal thermogram in Fig. 1A is an average obtained from plasma samples of 120 individuals. Fig. 1B shows thermograms for seven of the most abundant pure plasma proteins, normalized on a g/L concentration scale. The observed experimental thermogram is the vector **T** with excess specific heat values measured over a range of temperatures. Deconvolution of **T** requires fitting to a vector of concentrations **C** using a matrix of pure reference proteins **R**. **R** is a rectangular matrix of excess specific heat capacities at each temperature for each protein component. The classic least-squares solution to this problem is given by

$$\mathbf{C} = \text{inv}\ (\mathbf{R}' \times \mathbf{R}) \times \mathbf{R}' \times \mathbf{T}$$

where $\mathbf{R}'$ is the transpose of **R**. The experimental thermogram can be fit to a minimum of five protein components, as shown in Fig. 1A. The deconvoluted contribution of human serum albumin (HSA), immunoglobulin G (IgG), fibrinogen, immunoglobulin A and haptoglobin are shown in Fig. 1A, along with the residual plot inset at the top of the panel. Attempts to include more than these five proteins led to nonsensical fits characterized by negative concentration values. This indicates that only a limited number of unique component thermogram shapes can be resolved that contribute to the observed experimental thermogram. The experimental thermogram is dominated by contributions from HSA and IgG, but contributions from other components are needed to account for subtle features of the thermogram.

The power of DSC in characterizing binding interactions is exploited in its application to the study of the disease-state plasma interactome. Putative interaction of disease markers with the more abundant plasma proteins is akin to ligand–protein binding for which detailed protocols are firmly established for the analysis of ligand-induced shifts in denaturation thermograms. Changes in the thermogram as a result of binding interactions can be dramatic and can be related quantitatively to the binding constant and binding enthalpy of the biomarker. DSC thermograms have potential utility as a diagnostics tool because they are sensitive to changes in protein composition both in a non-interacting mixture and as a consequence of modifications or interactions with other components. An example will be shown later to illustrate this.

The aim of this article is to document necessary considerations for obtaining reproducible thermograms. An example of the utility of the approach will be provided.

3. Materials and methods

In order to obtain consistent, reproducible plasma thermogram, specific protocols must be zealously followed. A detailed, step-by-step standard operating procedure for obtaining thermograms is provided as Supporting information. Additional comments relevant to the data presented follow.

3.1. Samples

DSC is considered a universal detector and as such is applicable to a wide array of samples. Table 1 is a testament to the versatility of DSC for the analysis of clinical samples. The majority of publications highlighted in Table 1 focused on the analysis of blood plasma or serum samples but the analysis of a range of other biological samples has been reported, including cerebrospinal fluid and digested tumor samples. This article will focus on DSC analysis of plasma/serum samples. Plasma/serum samples were purchased commercially for our studies, for example, pooled healthy control plasma was obtained from Sigma–Aldrich (St. Louis, MO) and used for the experiments examining pre-analytical and analytical factors on plasma thermograms (Section 4.1). Single donor healthy plasma was obtained from Innovative Research (Southfield, MI) and formed the majority of samples in our healthy control group. A large number of samples, including additional healthy controls as well as the majority of the disease samples we have analyzed, have been obtained through clinical collaborations. In this event, it is necessary to ensure that the studies undergo review and approval by the appropriate Institutional Review Boards before commencing and that all human subjects protocols and biosafety