Scanning Complex Biological Samples

Keywords: differential scanning calorimetry, diagnostic, serum, cancer, brain tumor

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ABSTRACT

Thermal stability signatures of biological fluids, comprised of a complex milieu of protein can be measured using differential scanning calorimetry (DSC). The DSC is a powerful tool in monitoring changes in the protein equilibrium that is perturbed by disease or cancer through amplification, modification, or multiple interactions of proteins and peptides. Through thermal denaturation of the proteins in a body fluid or tissue, trends have emerged that are linked to disease type and its progression, making DSC a complement to historical diagnostic techniques.

INTRODUCTION

THE PLASMA PROTEOME

The proteome is comprised of ~ 3000 proteins with over 90% of the protein content in plasma comprised of only 12 types (Figure 1) (1). Abundant biological macromolecules such as serum albumin and immunoglobulins that are currently used in an FDA approved test as elevated or suppressed levels can indicate diseases (2-4). It is hypothesized that the smaller peptides and proteins that contribute to a lesser proportion could potentially contain hundreds of interesting biomarkers. Initial success at proving this hypothesis has been achieved with particle size and charge techniques, such as, 2D electrophoresis and mass spectrometry (5-8). However, this success has not been without roadblocks. For some cases, the diagnostic peptide is not detectable due to low concentration or complexation. This complex web of multiple interactions that includes biomarkers and the more abundant proteins like HSA or IgG is referred to as the "interactome" (9).

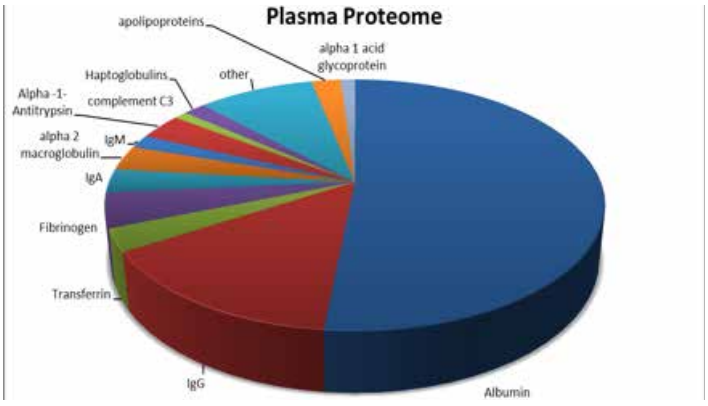

Figure 1: Major Plasma Proteins. Figure was created based on average concentrations: Putnam (1).

DSC

By measuring the difference in heat flow between a sample and a reference, while both are heated at a constant rate, differential scanning calorimeters are able to measure the amount of heat absorbed or released during thermally induced transitions. An event in one cell creates a thermal imbalance that is compensated for by feedback heaters attached to the instrument cells. In most experimental biopolymer DSC studies, a single pure protein is scanned against a buffer while the thermally induced enthalpy of unfolding, temperature of unfolding, and change in heat capacity is collected. An additional application includes quantification of the binding affinity between a ligand and a target macromolecule (10).

Investigation of a complex milieu is an emerging field of study using a DSC instrument. Instead of a single protein, multiple proteins in a single sample of plasma, urine, cerebral spinal fluid, and brain tissue homogenates are assayed with a DSC (11-22). DSC is sensitive to composition and interactions within a sample and a complicated thermogram is related to complexity of the samples. Variations detected include multiple domains, intermediates formed, possible oligomeric states, and impurities. For serum, tissue, and other samples containing multiple proteins, the overall thermogram is the sum of each individual plot, where the magnitude is correlated with its abundance in the sample. Furthermore, any binding to these proteins in this complex will alter the thermogram as well. If the binder stabilizes the overall globular protein structure, then the T_m will increase, and if the binder is specific for a site only available in the unfolded state, then the T_m will decrease.

EXPERIMENTAL

The majority of the publications to date use the same simple procedure: centrifugation, filtering, dialysis, and dilution (~ 2 mg/mL total protein for plasma yeilds a suffcient signal). Addition of DNAse and collagenase has been used as well for tissue samples (Chagovetz. et al.). A solution such as phosphate buffered saline at physiological pH is used to dilute the samples. The sample is scanned on a senstive DSC, such as the NanoDSC, at 0.5 to 2 ˚C/min. A slower rate is chosen for fine detail definition and a faster rate for a larger signal. The resulting thermogram is normalized to the concentration of total protein that has been determined via the biuret, bicinchoninic acid assay or a similar method. An advantage to using DSC in comparison to other techniques is that it does not require labels or complex fractionation.

A background buffer scan is subtracted and the data is normalized - typically to the total protein content. The resulting unfolding thermogram can be fitted and the T_m or T_{max}, ΔH, and shape are unique to each protein under a specific condition.

PLASMA AND SERUM STUDIES

When investigating the proteome with DSC, the first thermogram to establish is the "normal". This can be normal serum, plasma, or tissue, depending on the sample source. One of the reasons that this type of DSC experiment works is consistency. The thermograms below in Figure 2 were collected and assayed in a similar manner to those outlined by Garbett et al. 2009 (14). The profile agrees with previous data of 100 healthy individuals (14) and as expected, the fibrinogen peak that is present in plasma (data not shown) around 50 ˚C is absent in the serum thermogram. The normal or healthy assay is often used to assess the collection and processing of all experimental samples.

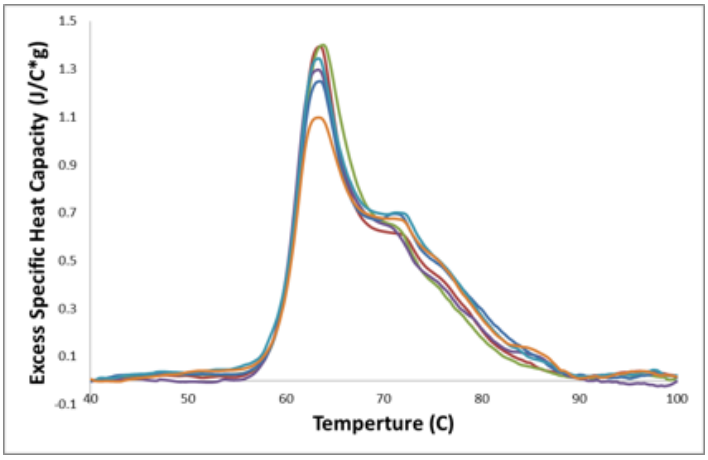

Figure 2. Normalized serum thermograms from three healthy males and three healthy females.

One of the plasma thermograms from this group was plotted along with the individual thermograms of a few of the more abundant proteins in plasma (Figure 3). The individual protein thermograms were normalized using the area under the plasma thermogram, and this area was multiplied by the percent protein expected in plasma. These four abundant proteins in the blood make-up the majority of the features of the experimental thermogram and enable identification of the major peaks. A more thorough study including the 16 most abundant proteins was completed earlier and the additional proteins add the fine detail to the thermogram (13, 14).

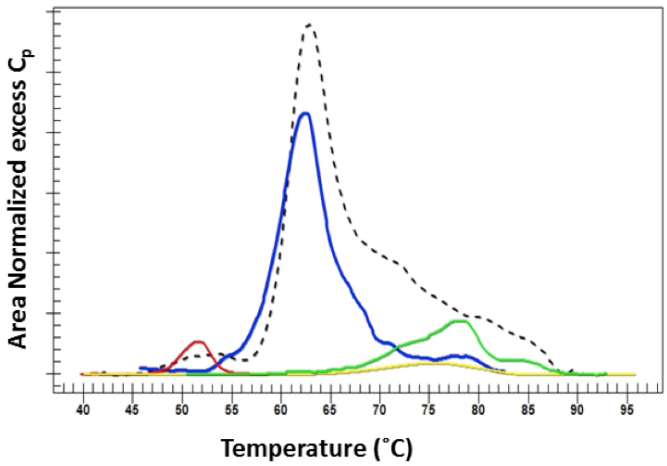

Figure 3: Dashed black line: individual plasma sample. Red: fibrinogen, blue: serum albumin, green: IgG, and yellow: IgA.

QUANTIFYING CHANGES IN MAGNITUDE

There was a distinct difference in all disease versus normal plasma thermograms cited in this body of work (11-15, 17-19, 21, 22). Most noticeably was an increased percentage of the area of the total thermogram originating from higher temperature transitions. In order to better explain this shift, the idea of citing the temperature at half the area under the entire unfolding event (temperature first moment, T_{FM}) was introduced (13) and has been adopted by others (19). In Figure 4, a normal plasma thermogram has both the T_{max} and T_{FM} marked with excess heat capacity (C_p^{ex}) on the y-axis. A common feature of the thermograms is a decrease in the magnitude of the T_{max} and an increase in the later, higher temperature peaks, thereby shifting T_{FM}. This could be due to relative changes in the concentrations of different proteins, such as the albumin concentration has decreased while the immunoglobulin concentration has increased. An alternative to this theory is proposed later in "modification and the interactome".

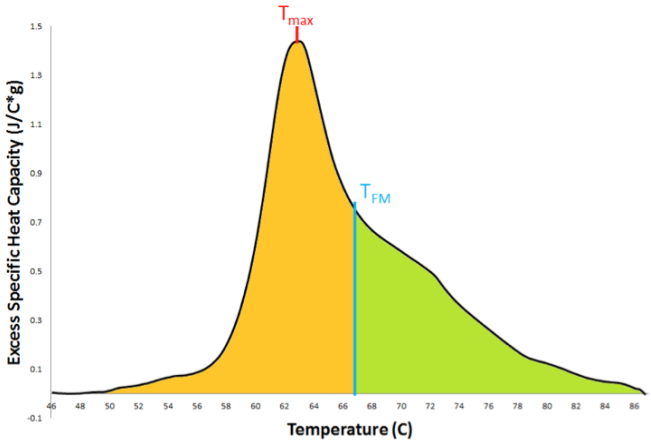

Figure 4. Features of unfolding: the yellow is the first half of the total area and the green is the second half of the total area.

For soft tissue inflammmation (STI) there was a decrease in the albumin concentration and an increase in the immunoglbulin and fibringogen concentrations that was observed in the thermogram (3). The T_{max} did not change for these cases, but the T_{FM} did shift to a higher temperature as the concentration of the higher temperature proteins, such as the immuoglobulins, increased (19). Thermograms from patients with Systemic Lupus Erythematosus were also distinguished from the healthy profile because of the increases in haptoglobulin, IgA, and IgM concentrations (13).

In addition to the inflammation study, the primary focus of the Todinova paper was to assay colorectal cancer patient plasma. It was important not only to study healthy versus neoplastic thermograms, but also inflammation versus cancer, because inflammation can be a "hallmark of cancer" (19). The STI thermodynamic profile was distinguishable from the cancer profiles. Within the cancer examples, groups and subgroups were thermally identified using parameters such as T_{FM}, and excess heat capacity (C_p^{ex} similar to height of peak) ratios of identified peaks. This study opens the idea that the difference in profiles was not because of a decrease or increase in protein levels, but had originated from another cause other than amplification.

For the separate diseases of chronic obstructive pulmonarydisease (COPD) and breast cancer, the thermograms of plasma showed significant changes in the albumin peak. This peak appeared to be either shifted to a higher temperature or the level of albumin was decreased (15,18). Based on the finding of Todinova, it is more likely that the protein concentrations remained consistent and that there was another protein stabilizing effect occurring. As the diseases progressed through stages, there were observable changes within the thermograms. An increase in the T_m of the major peak within the thermogram being related to the progression of each of the illnesses (15,18). For the breast cancer study, the shift in the T_{max} was also related to the size of the tumor (18). The DSC showed some potential discriminatory power for both grade and stage in these two diseases.

MODIFICATION & INTERACTOME

The complex and webbed interaction between proteins and peptides has inspired the term "interactome" (9) Components of the peptidome bind with and thereby modify the more abundant proteins, such as those listed in Figure 1. One of the difficulties posed when exploring small biomarkers with serum protein electrophoresis (SPE), is the minor changes in both size and charge.

The interactome idea may explain the trends observed in the DSC profile for diabetic patients concurrently suffering with either severe coronary artery disease, rheumatoid arthritis, or Lyme disease (12, 13). The FDA approved SPE assay that detects changes in major serum proteins, did not detect any significant changes between normal and diseased samples for these three separate cases (12,13). However, the disease thermograms did display a marked change with larger magnitudes of higher temperature peaks (12,13). If the protein concentration of the albumin and IgG had changed in magnitude or charge, this would have been detected by SPE. The changes detected by the DSC could be indicative of a modification or interaction of the major serum proteins with a potential biomarker.

The hypothesis of the interactome was investigated by Garbett et al. 2008 by adding a known binder, bromocresol green, to a pure albumin sample (13). While the total protein content remained consistent, the peak shifted to a higher temperature, indicating that the addition of the ligand helped stabilize the folded form of the protein (13). This theory has been further supported by a recent study of type 1 diabetes patients with early renal function decline (21). Qualitatively, there was a significant shift in the size of the T_{max} peak relative to normal and an increase in the area percent of the higher temperature peaks (21). Thermal stabilization through post-translational modification could account for increase in higher temperature peaks as the levels of albumin and immunoglobulins remained constant. This idea was corroborated by an increase in modified or oxidized proteins that are known to interact with other proteins (21).

Recent studies have paired this qualitative idea of the interactome with a more quantitative approach that combined DSC and Mass Spectrometry (MS). To support this approach, the shift in the DSC profile of the T_{FM} to a higher temperature was due to modified proteins rather than an increase in globulins and a decrease in albumin. Different fractions were also analyzed by MS (22). In the fraction labeled FX2, defined as isolated peptides that were bound to total plasma proteins, several biomarkers in the plasma and urine were identified by MS, thereby supporting the interactome concept (22). The discriminating features of the DSC that enabled differentiating between cancer stages were observed in the C_p^{ex} (height) ratio (22). All types of cancer were significantly different from the healthy samples, showing discrimination in T_{max}, T_{FM}, width and C_p^{ex} ratios (22).

SAMPLE TYPE ALTERNATIVES

CEREBRAL SPINAL FLUID

One of the non-plasma examples, cerebral spinal fluid (CSF), from patients with brain cancer, was chosen because it would be a closer fluid to the disease of interest (16). Chagovetz was able use DSC thermograms to definitively separate neoplastic and non-neoplastic cases, and within neoplastic cases, carcinomatosis meningitis originating from non-neural cancer from the neural cancer, glioblastoma multiforme (GBM) (16). A key observation was that the metastasized cancer retains specific properties that differentiate it even though it has spread to the meninges.

TISSUES

DSC was used to assay homogenates from human brain tumor biopsy samples and the resulting thermograms were correlated with histological tumor classification (20). The data

was fitted to four independent Gaussians with variables for temperature (T_m) enthalpy (ΔH) and a scaling factor (A_w). A_w is related to the relative abundance of the protein denatured in the transition. A_w and ΔH also describe the overall shape of the transitions. A small ΔH and large A_w will result in a relatively wide and short unfolding transition, whereas an unfolding transition with a small A_w and large ΔH will be relatively sharp. Differences in glioma-specific thermograms were described by using calculated parameters at transitions that were characterized and statistically significant differences typically observed in the A_w and ΔH parameters (20). These differences indicated that the temperature of unfolding didn't change significantly, but the abundance and ratios of proteins at these temperatures did.

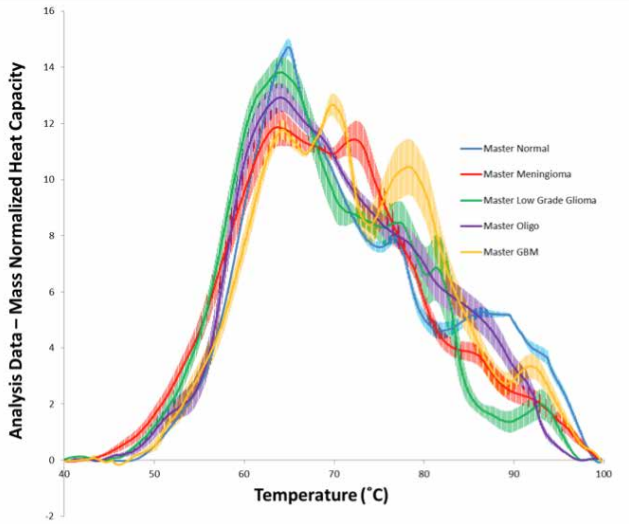

Figure 5. Average thermograms for brain cancer tissue and normal tissue samples.

CONCLUSION

The studies referenced in this review have successfully demonstrated a correlation between an observed unique thermodynamic signature collected from body fluids and tissues and an existing disease. With each malady having its own DSC signature, the DSC technique lends itself to becoming an essential and complimentary diagnostic tool in identifying and monitoring disease progression. The data collected via DSC should be considered a compliment to currently approved clinical methods such as SPE and histology and their ability to differentiate types and grades of diseases. A thorough and detailed differential diagnosis can be critical in the selection and administration of the most efficacious therapy. As more studies are completed, it is possible that a reference library of normal and disease DSC profiles will emerge and be utilized to aid in future cases.

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