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Measurement of protein denaturation in human synovial fluid and its analogs using differential scanning calorimetry

Lee-Ann K. Briere · Jan-M. Brandt · John B. Medley

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Abstract Differential scanning calorimetry (DSC) was used to evaluate the thermal transitions associated with protein constituents of synovial fluid samples from three individuals with osteoarthritis. Analysis of the multi-component DSC curves revealed that major endothermic transitions of synovial fluid occur between 60 and 80 °C and can be resolved into three peaks, likely due to the unfolding of human serum albumin and immunoglobulins, and that the enthalpies of these transitions can be quantified in terms of their relative contribution to the total system enthalpy. DSC was also used to analyze a solution of bovine calf serum, a lubricant used in simulator wear testing of joint replacement implants, and the resulting endothermic transitions occurred in a temperature range relevant to that produced by frictional heat during such wear simulator testing. Results of this study indicate a new application for DSC as a direct method for studying thermal stabilities of both bovine calf serum and synovial fluid. The use of DSC is proposed as a diagnostic tool to detect altered thermal properties or protein concentrations indicative of a diseased or injured state, and as a development tool to test the efficacy of additives in controlling protein denaturation associated with increased wear in joint replacement implants.

L. K. Briere (✉)
Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario, Medical Sciences Bldg Room 394, London, ON N6A 5C1, Canada
e-mail: lbriere2@uwo.ca

J.-M. Brandt · J. B. Medley
Department of Mechanical & Mechatronics Engineering, University of Waterloo, Waterloo, ON N2L 3G1, Canada

Keywords Differential scanning calorimetry · Synovial fluid · Serum albumin · Protein stability · Thermal unfolding · Osteoarthritis

Abbreviations

HSA	Human serum albumin
BSA	Bovine serum albumin
DSC	Differential scanning calorimetry
ACS	Alpha calf serum
PBS	Phosphate-buffered saline

Introduction

Synovial fluid (SF), the biological lubricant of human synovial joints, is a complex mixture of blood plasma proteins (albumin and several types of globulins), lipids, hyaluronic acid, proteoglycan, and many small molecules such as glucose, salts, and carbon dioxide. Together, the subchondral bone, the natural cartilage that covers the joint, and the SF act together to provide a low-friction, low-wear environment that is normally in homeostasis [1, 2]. Upon injury and diseases such as osteoarthritis, however, progressive damage occurs to the synovial joint surface, and this is accompanied by SF composition changes [3].

Severe wear of the natural cartilage that occurs in end-stage osteoarthritis is usually best treated by total joint arthroplasty (replacement of the articulating surfaces with artificial materials). A major difficulty encountered in total joint arthroplasty is wear (erosion) and, more specifically, the associated wear particle induced osteolysis around the implant [4–6]. Thus, the in vitro wear evaluation of such artificial joint replacements has become an essential part of the product development process for total joint replacements

where protein-rich lubricants, such as different types of bovine calf serum, are used to mimic the lubricating properties of the SF [7–10].

The integrity of SF constituents, or any protein-rich lubricant constituents, depends to a large extent on the temperature in the contact zone of the joint. For joint replacement implants, increased temperatures lead to increased protein degradation and accelerated wear [10], so it follows that the heat generated at the interface of the joint surfaces due to asperity contact may play a role in the wear process [11]. Human serum albumin (HSA), the most abundant protein in SF, has been shown to have a significant influence on sliding friction and, therefore, is also likely to play an important role in the lubrication of cartilage and artificial joint materials [4, 12]. Similarly, bovine calf serum, currently the optimal choice as a lubricant for in vitro wear tests of total joint replacement implants, has bovine serum albumin (BSA) as the major protein constituent. BSA has been shown to protect against wear processes comparably to those occurring in prosthetic joints in vivo [7]. Although lubricant components such as mammalian albumins and γ-globulins have been studied extensively as individually purified proteins, and the effects of additives and factors such as pH on the thermal transitions of these proteins are known in some cases [13–23], there exists a need for a technique in which biophysical properties of SF and serum constituents can be studied directly within the context of the complete bio-lubricant.

Differential scanning calorimetry (DSC) is a sensitive technique employed for the highly reproducible detection and quantification of temperature-induced transitions such as protein denaturation [24–26]. As temperature is increased at a uniform rate, curves depicting excess specific heat (C_p) versus temperature are obtained, and the area of the heat absorption peak represents the calorimetric enthalpy of the unfolding process.

Temperature-induced structural changes of a macromolecule may proceed in one or more cooperative stages, often due to different structural domains of a protein. The presence of two or more domains within a protein results in a more complex denaturation profile that is identified by the presence of multiple transitions, often overlapping, shown as the sum of the transitions of all components [27]. More intricate profiles will arise from the analysis of complex biological samples containing multiple constituents, such as those seen for isolated cellular organelles and intact cells, which can contain in excess of 6 to 8 major transitions in a temperature range from 30 to 95 °C [25, 28–31]. Unlike the scanning of dense solutions of intact cells, which contain far more proteins and additional events such as metabolism, lipid and DNA transitions, SF is comparatively simple and therefore should have a less complex, more easily interpretable DSC profile. Each component of SF may be expected to undergo its characteristic endothermic transitions and, providing that these transitions occur at different temperatures, the major transitions can be studied over a wide temperature range in the natural context of the mixture. This allows for these important properties of natural and artificial joint lubricants to be determined, to evaluate thermal stability, and to use these findings as a tool in the design of artificial lubricants with a performance that matches or exceeds the natural lubricants [4].

The purpose of this study was to use DSC to investigate the endothermic transitions observed in osteoarthritic human SF as a means of analyzing protein content and denaturation. The thermostabilities of proteins in the SF of three different osteoarthritic patients were compared. A preparation of alpha calf serum (ACS-I), a common lubricant for simulator wear testing of joint replacement implants, was also analyzed. Presented here are baseline data demonstrating that DSC provides a new method to characterize the proteome of human osteoarthritic SF and bovine serum lubricants used in wear testing of joint replacement implants.

Materials and methods

SF samples (SF1, SF2, and SF3) were obtained from patients with osteoarthritis as their primary diagnosis, during their primary knee replacement surgery. Ethics approval was obtained from the Research Ethics committee at the University of Western Ontario (Approval Number 12536E). SF samples were subjected to serum protein electrophoresis on a commercial agarose screening-gel (Sebia Hydrasys Hydragel 30 β1–β2, Sebia, Georgia), to resolve the five serum proteins (albumin, α-1 globulin, α-2 globulin, β-globulin, and γ-globulin). Samples were then assayed for total protein content using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, Illinois), and concentrations were determined to be 32.2, 29.8, and 43.1 g/L for SF1, SF2, and SF3, respectively. Iron-supplemented alpha-calf serum samples (ACS-I) were obtained from HyClone (Lot# AQE23894, Logan, Utah) with a total protein concentration of 41 g/L. All samples were frozen for storage prior to measurement.

Differential scanning calorimetry

All DSC analyses were carried out in triplicate in a VP-DSC instrument (MicroCal, Northampton, MA, USA). Immediately before injection into the calorimeter, aliquots of SF samples were thawed. It was found that undiluted SF samples were too viscous for effective filtration and degassing, so they were diluted with phosphate-buffered

saline (PBS) to a total protein content of 6 g/L, filtered with a 0.8 μm Pall Acrodisc (Pall Corporation, Ann Arbor, Michigan), and degassed by gentle stirring under vacuum prior to analysis. ACS-I samples were treated similarly and diluted with PBS to a concentration of 6 g/L.

Differential scanning calorimetry curves were generated for samples over a range from 10 to 90 °C, at a scan rate of 60 °C/h. Buffer–buffer reference scans were recorded under the same conditions and subtracted from sample endotherms. Raw data were processed by nonlinear least squares regression analysis in Origin 5.0 (MicroCal), by first subtracting a buffer–buffer reference scan and then fitting to a non-two-state transition model.

Data analysis

Multi-component biological solutions (or solutions of proteins with a number of independent structural domains) undergo multiple transitions and hence, exhibit curves with complex profiles. Therefore, for each protein in solution (X, Y, Z, etc.), or structural domain of a protein, undergoing a transition between a folded and unfolded state ($X_f \leftrightarrow X_u$, $Y_f \leftrightarrow Y_u$, etc.), equilibrium constants can be expressed in fractional terms of $K_X = \frac{f_{X_u}}{f_{X_f}}$, $K_Y = \frac{f_{Y_u}}{f_{Y_f}}$, etc. [24, 32, 33]. In such complex systems, the curve represents the sum of these numerous order–disorder transitions, and the total molar enthalpy of the system, H, which is equal to the area under the DSC curve, is then represented by:

$$H = H_F + f_{X_u}\Delta H_X + f_{Y_u}\Delta H_Y + \text{etc.} \quad (1)$$

where H_F represents the initial form of all components of the sample, when all proteins are in their folded state, and ΔH_X, ΔH_Y, etc., represent molar enthalpy changes of the individual proteins and/or their structural domains. Total molar heat capacity of the system, C_p, would be similarly calculated from the sum of C_{pF} (the molar heat capacity of the fully folded states) and the corresponding fractional heat capacities of the individual proteins and/or their structural domains [24].

Before all curve fitting, a generated baseline was subtracted to remove ΔC_p effects, rendering both C_{pF}, the molar heat capacity of the totally folded state, and ΔC_p, to zero at all temperatures. It should be noted that when the data was fit to a model that gave a non-zero ΔC_p, very poor fits were obtained. The model that best fit the data was an independent non-2-state model, defined by Eqs. 2 and 3. Also, in the absence of a specific method to determine the exact concentration of each individual protein component of SF and therefore their relative contribution to the endothermic signals, the ΔH values obtained lacked quantitative thermodynamic significance and were therefore used only in relative (ΔH_{rel}) terms.

$$C_p(T) = \frac{K_X(T)\Delta H_{X_{rel}}}{(1+K_X(T))^2 RT^2} + \frac{K_Y(T)\Delta H_{Y_{rel}}}{(1+K_Y(T))^2 RT^2} + \frac{K_Z(T)\Delta H_{Z_{rel}}}{(1+K_Z(T))^2 RT^2} + \text{etc.} \quad (2)$$

$$K_X(T) = \exp\left[\frac{-\Delta H^*_{X_{rel}}}{RT}\left(1 - \frac{T}{T_{mX}}\right)\right] \quad (3)$$

where ΔH^* is the van't Hoff heat change for the reaction, which corresponds to the heat change for the cooperative unit or protein domain that participates in the reaction, and T_m is the temperature at which C_p reaches its maximum value [34].

Results and discussion

Human osteoarthritic SF

In an unprocessed DSC plot of C_p versus temperature for SF samples taken from three different individuals with osteoarthritis, no transitions were seen below 55 °C but a series of endothermic transitions occurred between 60 and 80 °C (Fig. 1). An intrinsic curvature in the baseline due to instrumental effects was seen, but was corrected for when the buffer scan was subtracted.

Macromolecules present in SF include hyaluronan, proteoglycan 4, surface-active phospholipids, HSA and various globulins, typically at concentrations of 1, 0.05–0.35, 0.1, 11 and 7 mg/mL, respectively [1–3]. Given the relative abundance of each component, it was expected that the major transitions in the DSC curves would represent primarily the denaturation of the polypeptides [27], as has been definitively shown for the DSC transitions of human blood plasma [27, 35]. Examination of our data suggested 3 overlapping transitions, and fitting to a 3-component

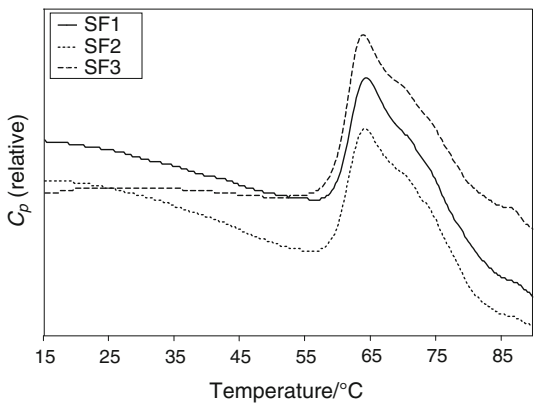

Fig. 1 DSC curves of human osteoarthritic synovial fluid (SF). Plot of C_p vs. T of 3 different SF samples, obtained after scanning to 90 °C at a rate of 1 °C/min

system provided very reasonable fits across the individual scans, except at the very high end, where deviation from the curve was least significant. The profiles of Fig. 1 were deconvoluted into three major peaks, each contributing to the endothermic signal in the 60–80 °C range (Fig. 2a–c). The melting temperatures (T_m) and contribution of each transition to the total enthalpy were summarized using an independent, non-two-state model (Table 1). Relative enthalpic contributions were obtained by integrating the area under the deconvoluted curves and dividing them by the total area. The values obtained for the T_m of each peak were found to be very reproducible when performed in triplicate and were similar between the test subjects, although it appeared that the relative concentrations of the components causing the transitions did vary between patients. Also, since the extent of protein denaturation at any temperature can be approximated by the fractional calorimetric enthalpy, profiles from Fig. 2a–c were integrated and normalized to estimate the amount of fractional protein denaturation (f_D) for each temperature [27], and then plotted in Fig. 2d. While these profiles were similar between the three samples, it appeared as though the constituents of SF2 and SF3 were modestly more thermostable than those of SF1.

From the published DSC curves of Garbett et al. [35] for purified plasma proteins, the first peak of the curves has been identified as HSA. Pico [16], Farruggia and Pico [19], Tavirani et al. [14] and Michnik et al. [36] have also shown the thermal-induced denaturation of purified HSA using optical techniques and DSC measurements, at different pH values and scan rates. Corresponding well with T_{mA} of our results, Pico [16] showed that at pH 7.5 and the same scan rate of 1 °C/min, HSA exhibits a T_m of 63.2 °C, and that this T_m shifts or splits into two peaks when the pH is altered. The second and third peaks of the curves, occurring at approximately 70 and 75 °C, respectively, are likely due to the different globulin forms (α-1, α-2, β, and γ), which have been shown to contribute 2–3 transitions in this temperature range[21, 35]. Also, the relative contribution of the various globulins to the DSC signal is consistent with their typical concentrations in SF.

Interestingly, the curves from our SF samples exhibited profiles similar to those of human plasma, in recent work done by Garbett et al. [35, 37], who have shown that DSC profiles of plasma samples from healthy individuals can differ significantly from those obtained from patients with different diseases. The similarity between profiles is

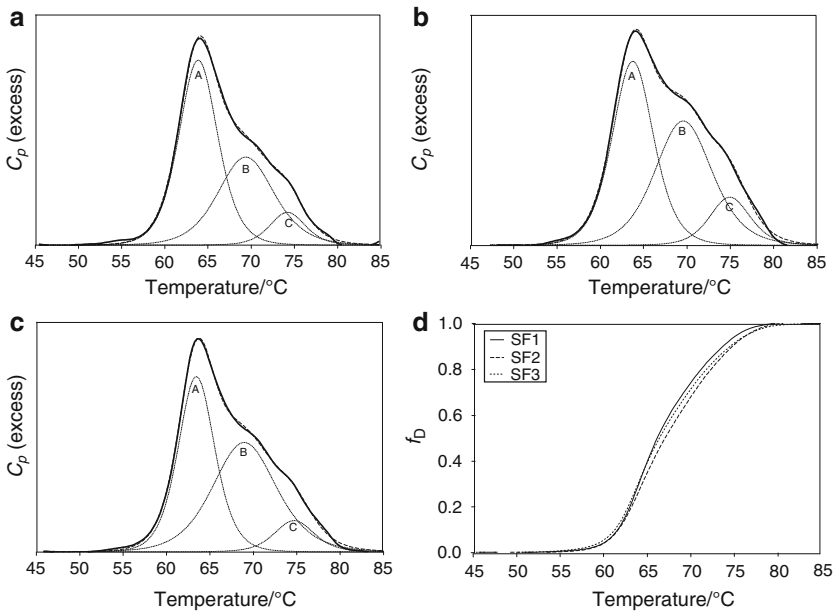

Fig. 2 DSC analysis of the denaturation of human SF from patients with osteoarthritis. Corrected plots of C_p (excess) vs. T (*solid line*) from 3 different individuals **a** SF1, **b** SF2, and **c** SF3 and subsequent deconvolution of transitions *A*, *B*, and *C* (*dotted lines*) contributing to the large endothermic transition seen in Fig. 1. The *dashed line* corresponds to the sum of these individual transitions. **d** Plot of fractional denaturation (f_D vs. T) obtained by normalization and integration of plots (**a**–**c**)

Table 1 Temperature midpoint (T_m) values of the three overlapping transitions, and relative contribution of each transition to the total enthalpy, observed in the DSC profiles of synovial fluid samples from three test subjects

Sample	T_{mA}/°C	Relative abundance/%	T_{mB}/°C	Relative abundance/%	T_{mC}/°C	Relative abundance/%
SF1	64.13 ± 0.34	36.15 ± 2.36	69.25 ± 0.57	46.60 ± 3.82	74.56 ± 0.79	17.42 ± 6.29
SF2	63.58 ± 0.10	41.39 ± 5.71	69.48 ± 0.36	45.39 ± 5.11	75.26 ± 0.49	14.07 ± 2.35
SF3	63.36 ± 0.15	42.04 ± 1.82	68.89 ± 0.14	49.07 ± 1.54	74.59 ± 0.19	8.86 ± 0.59

Experimental values (±SE) were obtained from three replicates per sample, followed by deconvolution using a non-two-state model

expected, as albumin and the various globulins are the most abundant proteins in both plasma and SF, and concentrations of glucose, lactic acid, carbon dioxide, nitrogen and salts are almost identical [38].

Alpha bovine calf serum (ACS-I)

To apply DSC to another system, this method was used to analyze a preparation of ACS-I, a common component of artificial lubricants. An unprocessed DSC profile (Fig. 3a) of an ACS-I sample heated from 10 to 95 °C was deconvoluted and analyzed. The largest endothermic signal could be separated into two peaks (Fig. 3b) with maxima at 60 and 63.5 °C, and is attributed to BSA, the largest protein component of ACS-I. The T_ms of these peaks correspond with the published T_m values of purified BSA, which has been reported by several groups to unfold in a manner dependent on scan rate and buffer composition in this temperature range [36, 39–43]. These studies have also shown that the shape and cooperativity of the DSC curve and the quantitative value of the T_m of BSA depend on the components of the solution in which it has been prepared. The two distinct endothermic peaks are attributed to the melting of independent domains of BSA due to the presence of a cleft in its structure [44].

One can also see the presence of a smaller peak in Fig. 3a at around 75 °C, which is likely from bovine gamma globulin, which has been shown in its purified form to denature at approximately 75.6 °C [45]. The source of the peak occurring at approximately 84 °C has not been identified. Fractional denaturation for the sample is plotted in Fig. 3c.

DSC for complex solutions

The DSC curves for osteoarthritic SF and ACS-I are similar to those reported for liver homogenate, cellular organelles, bacterial or mammalian cells and blood plasma [25, 27, 35], in that they consist of several transitions with variable ΔH and by a reproducible, characteristic distribution of T_ms defining the general shape of the profile.

It should be noted that despite a lack of exothermic transitions that can be associated with aggregation in any of the profiles shown here, in all cases calorimetric transitions for both SF and calf serum were found to be irreversible as indicated by the absence of an endothermic effect on a second scanning of the sample after cooling (data not shown). Furthermore, samples extracted from the calorimeter after a first scan showed visible precipitation.

Interestingly, joint implant wear simulators have shown, via repetitive articulation, that lubricant proteins precipitate during the elevated temperatures that arise from constant motion [4, 7, 12]. This is not surprising, given that temperature increases of up to 90 °C have been observed in joint simulator tests [7, 12], and large increases in heat often correspond with irreversible unfolding of proteins, as is seen in our DSC trials. Although less dramatic physiologically, it has been found that below the cartilage surface in vivo, temperatures of articulated artificial joints may rise to as high as 46 °C, and it is speculated that even higher temperatures that would induce the thermal denaturation of albumin could be reached at the interface [12]. Inside the joint, joint simulator or DSC cell, unfolded proteins exposing their usually buried hydrophobic residues preferentially adsorb onto hydrophobic surfaces or aggregate with other unfolded proteins and precipitate. The presence

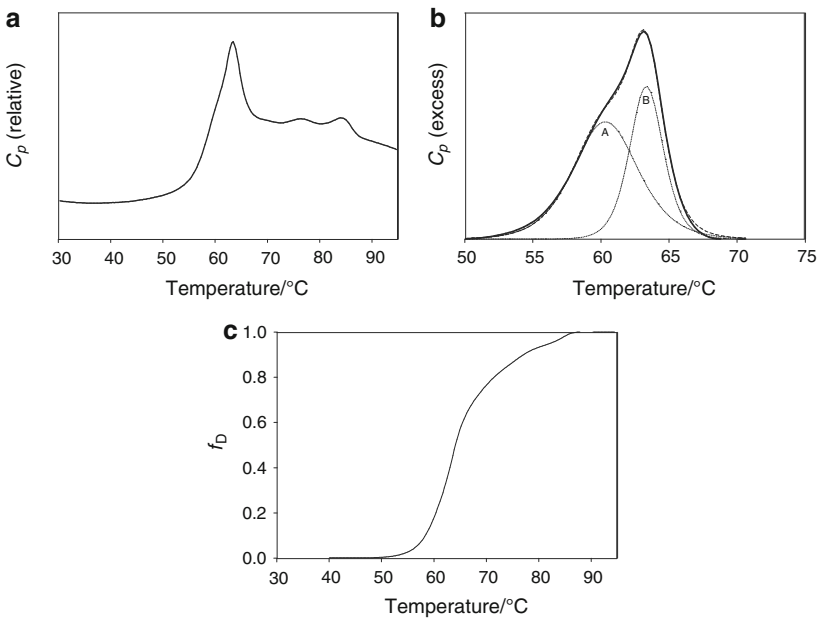

Fig. 3 DSC analysis of alpha bovine calf serum. **a** Plot of C_p vs. T obtained after scanning from 10 to 95 °C at a rate of 1 °C/min. **b** Corrected plot of C_p (excess) vs. T and subsequent deconvolution of transitions (*dotted lines*) contributing to the largest transition seen in (**a**). The *dashed line* corresponds to the sum of these individual transitions. **c** Plot of fractional denaturation (f_D vs. T) obtained by normalization and integration of (**a**)

of these precipitates adversely affects the lubricating properties of SF and lubricant serum by reducing the boundary lubrication while increasing the friction and/or wear rates [7]. Therefore, on hydrophobic surfaces, a positive correlation exists between friction force and the local concentration of denatured proteins in solution [12]. Also, increased friction during articulation has been associated with altered SF composition, such as altered albumin concentration [4, 46]. Our findings suggest that DSC would allow for direct analysis of these effects of concentrations on transition midpoints.

As previously reported, HSA and BSA have been shown to undergo several well-organized structural changes involving the temperature-induced unfolding and separation of specific domains independent of the rest of the protein [47, 48]. It has also been shown in vitro that altering the composition of the albumin buffer with salts, non-electrolyte additives, sugars, polyols and certain amino acids will stabilize or destabilize albumin structure, and directly affect ligand-albumin binding [14, 15, 21, 43, 49–51]. In the calorimeter, these alterations would be readily observed as an increase or decrease in melting temperatures, or as changes in the sharpness or cooperativity of the transitions. Relating this to the frictional heat generated in artificial joint articulation processes, adding stabilizers or protectants to SF or bovine serum lubricants could possibly alter the onset of denaturation of their constituent proteins and thus improve these lubricants' ability to reduce wear. This notion is supported by Heuberger's findings that the conformational state of HSA in the lubricating solution can modify the efficacy of HSA-mediated boundary lubrication on a hydrophobic polymer surface [12]. Furthermore, despite the complexity of biological solutions, the addition of a salt, sugar or polyol can still have a significant effect on protein stabilization. Lepock et al. have shown that the simple addition of glycerol or ethanol to intact Chinese hamster lung V79 cells directly affects the onset of protein denaturation, and other groups have reported significant effects of NaCl on the thermal denaturation of intact bacterial cells [27–29, 52]. With respect to the comparatively simple solutions of SF and bovine serum solutions, additives would produce characteristic changes in observed DSC curves relative to those from additive-free lubricants. Such thermodynamic signatures could therefore be exploited in the design and development of lubricant additives that effectively increase melting temperatures, and thus reduce the concentrations of precipitated proteins and hence, once again provide a way to influence the wear of natural and artificial joints.

While not possible within the scope of this study, it is postulated that SF curves from healthy test subjects could be compared with those of arthritic or injured patients as a clinical assay to detect altered thermal properties or protein concentrations indicative of a diseased or injured state. It has been shown that rheumatoid arthritis patients have higher than normal albumin concentrations in their SF [46, 53], and this change would be observed in a DSC profile by comparison of relative enthalpies from the multiple endothermic transitions similar to what was shown in Table 1. It has also been shown that SF composition and rheological behavior in a replaced joint are similar to the SF of the diseased joint before arthroplasty (since the synovium is generally retained or reforms after total joint arthroplasty, enabling the capsule to recover and maintain its SF generating ability) [3], but most importantly that both are quite different from healthy SF [54]. Furthermore, alterations not in concentration but to the thermal properties of albumin or immunoglobins that may arise from the binding of peptide and/or protein biomarkers unique to different diseased or injured states [55, 56], would be readily identified by calorimetry. This idea of using DSC as a clinical assay for arthroplasty patients runs parallel to recent studies which have shown that blood plasma from patients with lupus, Lyme disease, and rheumatoid arthritis do in fact yield characteristic DSC curves with respect to each other and those from healthy test subjects [35, 56, 57]. To explore this possibility, future studies should, therefore, compare SF curves from healthy individuals to those from diseased or injured individuals to define the characteristics typical of such states.

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