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Ex vivo instability of glycated albumin: A role for autoxidative glycation

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ABSTRACT

Ex vivo protein modifications occur within plasma and serum (P/S) samples due to prolonged exposure to the thawed state—which includes temperatures above −30 °C. Herein, the *ex vivo* glycation of human serum albumin from healthy and diabetic subjects was monitored in P/S samples stored for hours to months at −80 °C, −20 °C, and room temperature, as well as in samples subjected to multiple freeze-thaw cycles, incubated at different surface area-to-volume ratios or under different atmospheric compositions. A simple dilute-and-shoot method utilizing trap-and-elute LC-ESI-MS was employed to determine the relative abundances of the glycated forms of albumin—including forms of albumin bearing more than one glucose molecule. Significant increases in glycated albumin were found to occur within hours at room temperature, and within days at −20 °C. These increases continued over a period of 1–2 weeks at room temperature and over 200 days at −20 °C, ultimately resulting in a doubling of glycated albumin in both healthy and diabetic patients. It was also shown that samples stored at lower surface area-to-volume ratios or incubated under a nitrogen atmosphere experienced less rapid glucose adduction of albumin—suggesting a role for oxidative glycation in the *ex vivo* glycation of albumin.

1. Introduction

Many of the naturally occurring biochemical reactions in the plasma and serum (P/S) compartment of blood are not at equilibrium when blood exits the body [1]. In addition, exposure of P/S to air results in a dissolved oxygen concentration [$O_2(aq)$] of approximately 0.25 mM [2,3]—an $O_2(aq)$ concentration that is much higher than that found in the P/S compartment of blood *in vivo*, where oxygen is carried on hemoglobin inside red blood cells. Introduction of this relatively high dissolved oxygen concentration into P/S establishes numerous additional redox disequilibria that did not exist *in vivo*. As such, proper storage and handling conditions are critical for maintaining the quality of P/S samples (i.e., maintaining P/S in a state that accurately represents blood chemistry *in vivo*). In particular, exposure to the thawed state permits (bio)chemical reactions to run toward equilibrium—a state that, for many reactions, does not represent biomolecular status *in vivo*. The thermodynamic melting of ice in plasma commences at −27 °C [4,5]. According to the European Pharmacopoeia, plasma is not completely frozen until −30 °C [6,7]. (The 3 °C discrepancy is probably due to the fact that plasma does not exhibit simple eutectic behavior [4,8].) Thus, storage and handling of P/S samples for prolonged periods above their melting point of −30 °C may compromise sample integrity, producing inaccurate and, in turn, misleading results in clinical research.

Numerous studies have demonstrated molecular alterations associated with inadequate sample handling and storage [9–14]. A recent study by Pasella et al. found that plasma samples stored at various temperatures above −30 °C, including −20 °C, 4 °C, and room temperature (20–25 °C), showed an apparent variation in the abundance of certain plasma proteins over a two-week period [12]. A metabolomics study concluded that the concentrations of nearly 20% of the metabolites in serum samples are impacted by storage temperature [13]. Another study showed that prolonged storage of either whole plasma or isolated plasma DNA had a substantial degradation effect on the DNA measured by real-time PCR-based assays [14]. Betsou et al. have summarized other various

Abbreviations: P/S, Plasma and serum; Hb1Ac, Glycated hemoglobin; GA, Glycated albumin; TFA, Triflouroacetic acid; WAG, Weighted albumin glycation; pcT2D, Poorly controlled type 2 diabetic.

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biomolecules in P/S that can be altered *ex vivo* by poor handling and storage, ranging from simple ion concentrations to DNA and protein concentrations [11]. It is clear that poor storage and handling can affect all types of molecules in P/S samples [15].

In vivo, P/S proteins undergo glycation through the conjugation of a reducing sugar monosaccharide (i.e. glucose) to the protein (usually via a lysine residue side chain) (Fig. 1). This process proceeds through the formation of a reversible Schiff base and later intramolecular rearrangements occur to form a stable, covalently-bonded Amadori product, which eventually leads to the formation of advanced glycation end-products [16]. Protein glycation is an unavoidable process of metabolism *in vivo*. The degree to which it occurs is determined by the concentrations of protein and glucose available in P/S and can be measured through various methods, such as glycated hemoglobin (HbA1c) measurements, the fructosamine assay [17], or by measuring glycated albumin (GA) [18,19]. *In vitro*, dissolved oxygen has been shown to contribute to a process known as autoxidative glycation—a phenomenon that drives protein glycation above and beyond that observed in the absence of oxygen and which is likely to be relevant to P/S samples *ex vivo* [20,21].

In clinical laboratories, P/S storage time is generally kept to a minimum and therefore tends not to raise some of the potential integrity issues that it can in research laboratories, where samples may need to be stored indefinitely. Clinicians employ HbA1c to gather an overall picture of what average blood sugar levels have been over a period of a few months in diabetic patients. Little et al. investigated the stability of HbA1c in whole blood samples kept at various storage conditions and determined that HbA1c was stable at −70 °C. However, when samples were stored at −20 °C they were only stable for 3–57 days, depending on the method of analysis [22]. Another clinically relevant test to measure blood glucose levels is the fructosamine assay. Fructosamine reflects blood glucose levels over the previous 2–3 weeks and may be used to help a person with diabetes monitor and control their blood sugar. The fructosamine assay has limited clinical usage compared to that of Hb1Ac; for example, it is not recommended in cases where there are significant abnormalities of plasma protein concentrations (e.g., nephrotic syndrome, liver cirrhosis) [23]. However, like the Hb1Ac test, storage conditions play a role in the stability of fructosamine in P/S. In 1988 a study was completed showing that fructosamine in serum is not stable when samples are stored at −20 °C for long periods of time [24]. Since albumin is the most abundant protein in P/S, fructosamine is predominantly a measure of GA—though other circulating proteins such as glycated lipoproteins and glycated globulins do contribute to the total concentration of fructosamine [25]. The concentration of GA can be directly measured by several methods, including gel electrophoresis, enzymatic methods, colorimetry, and immunoassays [18,19,26,27]. HbA1c remains the primary test for protein glycation that is used to monitor diabetes; however, studies have shown that GA may be more reliable than HbA1c in certain instances—such as specific clinical conditions in which HbA1c does not work properly (e.g., iron deficiency, pregnancy, and end-stage renal disease) [28].

As mentioned above, when P/S samples are drawn from patients they are exposed to a dramatic increase in dissolved oxygen concentration. Considering that this spike in oxygen concentration may contribute to autoxidative glycation, this creates the potential for substantial increases in protein glycation as P/S samples are exposed to the thawed state. In previous work our laboratory has analyzed the effect of storage and handling conditions on *ex vivo* protein oxidation, showing that exposure of P/S to temperatures above −30 °C can have a substantial impact on the oxidation status of albumin and apolipoprotein A-I—two of the most abundant proteins in P/S [9]. The simple dilute-and-shoot LC-MS method used in this study provided easily interpreted mass spectra for the relative quantification of all intact albumin proteoforms. Here, we implement this simple method to document how albumin glycation changes *ex vivo* under suboptimal storage and handling conditions and evaluate the impact of oxidative processes on the *ex vivo* glycation that takes place within P/S.

2. Materials and methods

2.1. Reagents

Trifluoroacetic acid (TFA; Cat. No. 299537) and formic acid (06440) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (L-16923) and HPLC grade water (L16978) were purchased from ThermoFisher Scientific (Waltham, MA). Hands-in-Bag atmospheric chamber (Cat. No. 15552-192) was purchased from VWR. Glucose Colorimetric assay kit (Cat. No. 10009582) was purchased from Cayman Chemical. Compressed oxygen and nitrogen were obtained from Arizona State University Stores and were 99.999% and 99.99% pure, respectively.

2.2. Blood plasma and serum

For the poorly controlled type 2 diabetic plasma sample involved in the multi-month time-course study (Fig. 3), fasting EDTA blood plasma was collected via venipuncture, under institutional review board (IRB) approval at the University of Southern California. The sample was received frozen on dry ice. To avoid potential acidification of the plasma sample, vial headspace was cleared of carbon dioxide prior to thawing for aliquoting [29]. Fifty-microliter aliquots were placed into each of three different types of vials for the long-term stability study at −20 °C: a cryogenic storage vial with inner threads and a sealing o-ring, a 1.5-mL snap-cap Eppendorf test tube, and a 1.5-mL snap-cap Eppendorf test tube with a ~2 mm hole punched in the top.

Additional matched, fasting EDTA plasma and serum samples were collected from several healthy volunteers, via venipuncture,

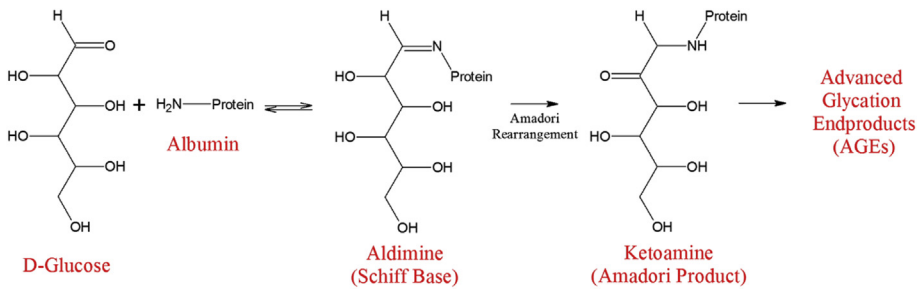

Fig. 1. Reaction scheme for the formation of glycated albumin. The non-enzymatic reaction between glucose and albumin starts with formation of a Schiff base. This is followed by an intramolecular rearrangement that leads to an Amadori product, which can ultimately lead to advanced glycation end products (AGEs).

under institutional review board approval at Arizona State University. The samples were collected according the NIH's Early Detection Research Network blood collection standard operating procedures [30,31]. Within 35 min of collection, plasma samples were processed, aliquoted, and placed in −80 °C freezer; serum samples were placed at −80 °C within 95 min of collection. One or more of these P/S specimens were used for all experiments described below. Unless otherwise noted, aliquot volumes were 50 μL.

2.3. Measurement of plasma glucose concentration

Plasma glucose concentrations were measured using the glucose colorimetric assay kit from Cayman Chemical (Product No. 10009582), following the standard manufacturer-recommended protocol. A Thermo Scientific Multiskan GO Microplate Spectrophotometer was used to record sample absorbance values at 520 nm.

2.4. Incubation under nitrogen or oxygen

P/S samples were incubated in a chamber filled with either nitrogen or oxygen using the hand-in-a-bag setup. One sample was removed from the chamber at each time point. During removal of the samples from the chamber, a positive pressure was used to ensure that no air was able to enter the chamber. No attempt was made to sparge oxygen from the P/S samples prior to incubating them under the nitrogen atmosphere. This decision was made due to concerns about the overall effectiveness of such a procedure. Thus a limited amount of oxygen must have been dissolved in these samples prior to their introduction into the nitrogen chamber.

2.5. Sample preparation and analysis

P/S samples were prepared for the analysis of intact albumin by LC-MS and then analyzed as previously described [9]. In brief, P/S samples were thawed at room temperature, mixed and centrifuged at 13,000 g for approximately 1.5 min to sediment any particulates. A half-microliter aliquot was diluted into 500 μL of 0.1% (v/v) TFA. Five microliters of this solution were injected immediately using a Spark Holland Endurance autosampler in microliter pick-up mode and loaded by an Eksigent nanoLC*1D at a flow rate of 10 μL/min using 80% water and 20% acetonitrile with 0.1% formic acid onto a protein captrap configured for unidirectional flow on a six-port diverter valve. The captrap was then washed for 3 min with this loading solvent. The flow rate over the captrap was then changed to 1 μL per minute and a 5-min linear gradient of increasing acetonitrile concentration from 20% to 90% was employed to elute the proteins into the mass spectrometer. The captrap eluent was directed to a Bruker MicroOTOF-Q (Q-TOF) mass spectrometer operating in positive ion, TOF-only mode, acquiring spectra in the m/z range of 300–3000 with a resolving power of ~20,000 m/Δm full width at half-maximum. Electrospray ionization settings for the Agilent G1385A capillary microflow nebulizer ion source were as follows: end plate offset −500 V, capillary −4500 V, nebulizer nitrogen 2 bar, and dry gas nitrogen 3.0 L/min at 225 °C. Data were acquired in profile mode at a digitizer sampling rate of 2 GHz. Spectra rate control was by summation at 1 Hz.

2.6. Data analysis

As previously described [9], approximately 1 min of recorded spectra were averaged across the chromatographic peak apex of albumin. The electrospray ionization charge-state envelope was deconvoluted with Bruker DataAnalysis v3.4 software to a mass range of 1000 Da on either side of any identified peak. Deconvoluted spectra were baseline subtracted, and all peak heights were calculated, tabulated and exported to a spreadsheet for further analysis. Peak heights were used for quantification as opposed to peak areas because of the lack of baseline resolution for some of the peaks. Representative raw mass spectra and their corresponding charge deconvoluted forms are provided in Supplemental Figs. 1–2.

Albumin has multiple glycation sites; thus, each molecule of albumin may contain multiple glycation states (a maximum of four glycation events per albumin molecule was observed—see Supplemental Fig. 3). The distribution of albumin in each of these four glycation states can readily be determined via electrospray ionization MS. This degree of analytical clarity facilitates comprehensive molecular analysis of albumin glycation and is expressed here as weighted albumin glycation (WAG). To calculate WAG, the mass spectral peak heights of native (unmodified) albumin and of singly, doubly, triply, and quadruply glycated albumin are multiplied by 0, 1, 2, 3, and 4 respectively, before normalizing by the sum of these peak heights. The glycated *and* (simultaneously) S-cysteinylated albumin proteoform was not considered because at early time points the relative abundance of S-cysteinylated albumin is low, making determination of the glycated form of this oxidized proteoform difficult to distinguish from baseline noise. As S-cysteinylation increases during P/S exposure to the thawed state [9], glycation of the S-cysteinylated form becomes apparent. However, for consistency, only the non-S-cysteinylated form of albumin was considered in all calculations. Since S-cysteinylation does not impact glycation, including information from this proteoform would provide, at best, a duplicate measurement of albumin glycation.

All statistical analyses were conducted with GraphPad Prism 7.

3. Results

3.1. Analytical reproducibility and autosampler stability

The analysis of intact human albumin by LC-MS for relative quantification of its various proteoforms has been previously described [9,32]. However, to verify that this approach to measuring albumin glycation is reproducible, intra- and inter-day precision of the analytical method were evaluated. Plasma samples from three healthy males and three healthy females were analyzed in quadruplicate on three separate days. The average weighted albumin glycation (WAG) in these samples was 14%. The average intra-day precision of WAG (expressed as %CV) was 4.6%, and the total inter-day precision was 6.4%. For the studies described herein, samples were injected onto the LC-MS immediately following dilution in 0.1% TFA. Nevertheless, we thought it might be useful to evaluate the autosampler stability of the diluted samples. WAG was found to be stable for at least 1100 min (~18 h), which was the time required to run 96 samples (Supplemental Fig. 4).

3.2. Impact of storage conditions on albumin glycation

The charge deconvoluted electrospray ionization mass spectra of albumin from a healthy fasting donor are shown in Fig. 2. The black spectrum represents a fresh, never frozen sample in which WAG was 17%. The red spectrum represents an aliquot of the same sample stored at −20 °C for 60 days, after which time WAG increased to 23%. Raw spectra and tables corresponding to the charge deconvoluted spectra shown in Fig. 2 are provided as Supplemental Figs. 1–2.

An increasing abundance of glycated albumin in plasma can be seen over time when samples are stored at −20 °C and at room temperature (25 °C)—both in a healthy donor and in a poorly

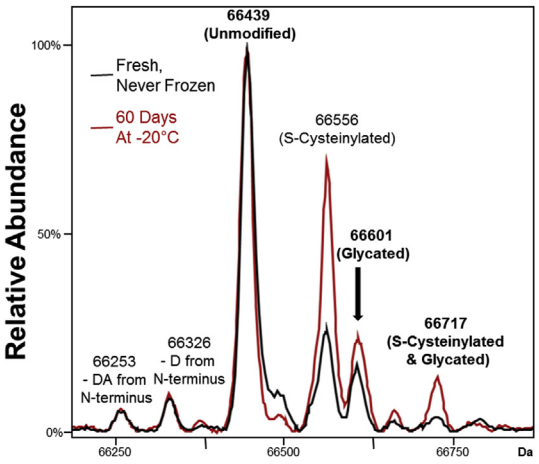

Fig. 2. Charge deconvoluted electrospray ionization mass spectra of albumin from a healthy donor showing an increase in glycated albumin under less-than-ideal storage conditions. The black spectrum is from a fresh, never frozen sample. The red spectrum is from the same sample stored at −20 °C for 60 days. *Ex vivo* changes in albumin S-cysteinylation (an oxidation reaction) under various storage and handling conditions have been described elsewhere [9]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controlled type 2 diabetic (pcT2D) patient (HbA1c > 10 and triglycerides > 1000 mg/dL, with a history of coronary artery disease and myocardial infarction) (Fig. 3A). Both samples were collected and analyzed fresh (i.e., at time 0) prior to storage at −20 °C. Free glucose concentrations (±S.D.) in the healthy and pcT2D donor were determined in triplicate and found to be 78.8 ± 0.63 mg/dL (4.37 ± 0.035 mM) and 194 ± 7.3 mg/dL (10.8 ± 0.41 mM), respectively. Analysis of glycated albumin at time zero revealed 17% WAG for the healthy donor and 28% WAG for the pcT2D donor. Each sample was then aliquoted into three different vial types and stored in a manual-defrost freezer at −20 °C (n = 3); an additional aliquot from the healthy donor was set aside for the room temperature time course. When stored at −20 °C, samples from both donors exhibited significant increases in the amount of glycated albumin, reaching 34% WAG for the healthy donor and 66% WAG for the pcT2D donor over a time span of approximately 200 days (Fig. 3A). At room temperature, albumin glycation increased within hours, reaching its peak within a week (Fig. 3B). Samples stored at −80 °C did not show a significant increase in albumin glycation (Fig. 3A).

When the individual data points from the pcT2D sample aliquots stored at −20 °C were separated based on the type of vial in which the specimens were stored, a statistically significant trend emerged in which the aliquot in the snap-cap test tube without a hole exhibited the smallest increase in *ex vivo* glycation, followed by the cryogenic storage vial with inner threads and a sealing o-ring, while the snap-cap test tube with a hole punched in the top exhibited the greatest degree of *ex vivo* glycation (Supplemental Fig. 5A; $p < 0.01$ for all three Wilcoxon matched pairs signed-rank comparisons. A Bonferroni correction for multiple comparisons set α at 0.017). These differences between storage vials were not as pronounced in the plasma specimen from the healthy patient (Supplemental Fig. 5B); in this case the only significant difference observed between the three data series was between the aliquots stored in the snap-cap test tube without a hole and in the snap-cap test tube with a hole punched in the top ($p = 0.0034$).

3.3. Matched plasma vs. serum

Matched sets of EDTA plasma and serum were collected from two healthy male and two healthy female donors to evaluate

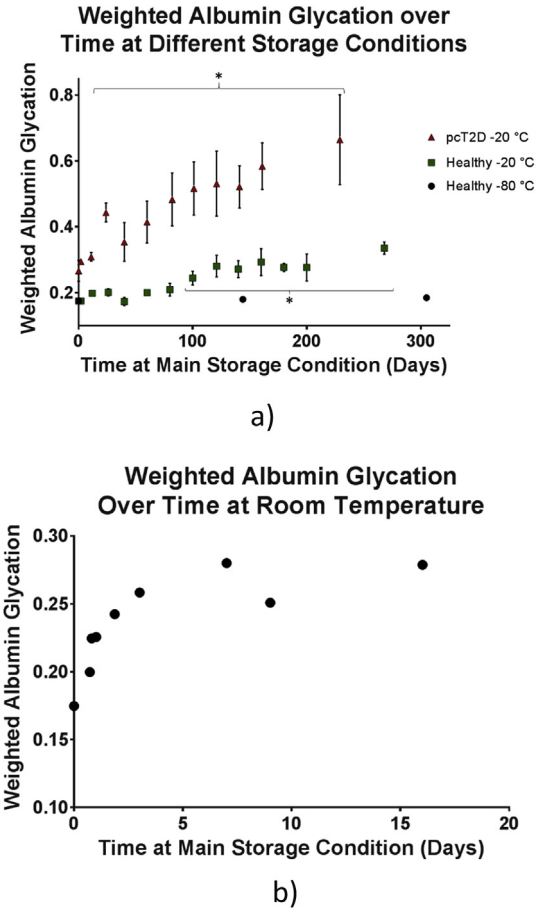

Fig. 3. *Ex vivo* increase in glycated albumin over time at different storage temperatures. WAG in plasma over time at −80 °C and −20 °C (manual defrost freezers) **(A)**, and at room temperature (25 °C) **(B)**. One sample was collected from a healthy donor and a second sample was obtained from a poorly controlled type 2 diabetic (pcT2D) with an HbA1c greater than 10 and a history of coronary artery disease and myocardial infarction. Both samples were collected and analyzed fresh, starting on Day 0—at which time WAG in the healthy and pcT2D samples was 17% and 28%, respectively. At −20 °C albumin glycation nearly doubled in both the healthy and pcT2D samples after about 200 days. Samples stored at −80 °C did not show an increase in WAG. Shown is the mean ± S.D. of the three aliquots per point. (* Indicates a significant difference from the initial time point; Repeated measures ANOVA, $p < 0.0001$ for all Tukey pairwise comparisons). An additional aliquot from the healthy donor was set aside for the room temperature time course. At room temperature **(B)**, albumin glycation increased within hours, reaching its peak within two weeks.

potential differences between plasma and serum regarding initial measurements of albumin glycation. Plasma and serum samples were processed and aliquoted within 35 and 95 min of collection, respectively, and then placed in −80 °C freezer until analysis. Average WAG ±S.D. in plasma was 17.7 ± 0.016; in serum it was 18.0 ± 0.015. A Wilcoxon matched pairs signed-rank test comparing plasma vs. serum revealed no significant difference in WAG between the two sample types.

3.4. Freeze-thaw cycles

To assess the effect of freeze-thaw cycles on albumin glycation, a plasma sample was collected from a healthy donor, measured immediately without ever being frozen (n = 6), then split into two separate vials. Both vials were then submitted to 20 freeze-thaw cycles (going from −80 °C to room temperature then back to −80 °C), with one vial subjected to opening and closing during each freeze-thaw cycle (to simulate aliquot withdrawal) and the

other vial remaining sealed during each freeze-thaw cycle. There was no significant difference in albumin glycation between the fresh sample and either of the freeze-thawed samples. Opening the vials during the freeze-thaw cycles (to simulate aliquot withdrawal) did not have a significant effect on albumin glycation (Fig. 4).

3.5. Surface area-to-volume effects

Because oxidation may play a role in *ex vivo* albumin glycation [21,33], the effects of surface area-to-volume (sa/vol) ratio on albumin glycation were investigated at room temperature for up to 25 days by dividing a fresh plasma sample from a healthy donor into 100-μL, 200-μL, and 400-μL aliquots in cylindrical, 8-mm internal diameter polypropylene screw-cap test tubes. Additional 10-μL aliquots were placed into a 1.5-mL conical-bottom polypropylene snap-cap test tube to represent an extreme case of high sa/vol. The effect of plasma sa/vol ratio on glycated albumin over time is shown in Fig. 5. Samples that were stored at a higher sa/vol ratio experienced significantly greater *ex vivo* glycation than those stored at lower sa/vol ratios for the same period. On day 1, the only sample that had a significant difference in WAG was the smallest volume of 10 μL (i.e., largest sa/vol ratio). For days 3 and 18 there were significant differences between all sample volumes. On day 11 there were significant differences between all samples except for between 400 and 200 μL. Day 25 there were significant differences between the 10 μL sample and all other sample volumes (Fig. 5A). Significance was determined using ANOVA; for all significant differences indicated, Holm-Sidak's pairwise comparisons $p < 0.05$. As plasma sample sa/vol ratio increases there is an increase in WAG (Fig. 5B). Plasma albumin glycation for all days was positively correlated to the sample sa/vol ratio (Pearson correlation, $r \geq 0.9$, $p < 0.05$).

3.6. Effect of atmospheric oxygen

To investigate the effect of atmospheric oxygen on *ex vivo* albumin glycation, aliquots of plasma from a healthy donor (collected on a different day than the other samples employed in this study) were incubated under nitrogen or oxygen for 16 days. For nearly all

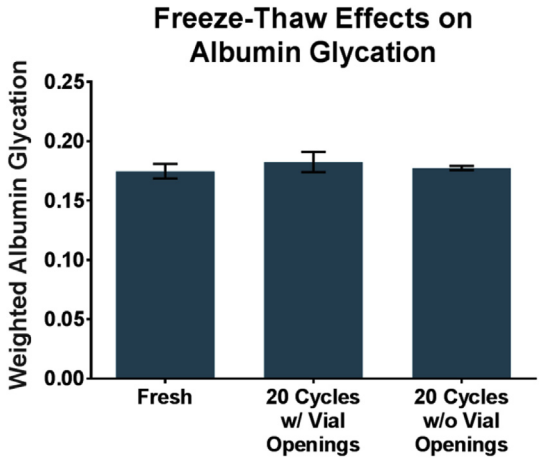

Fig. 4. Effect of freeze-thaw cycles on weighted albumin glycation. Following initial analysis (n = 6 technical replicates), a fresh plasma sample from a healthy donor was split into two cryogenic storage vials, each of which was subjected to 20 freeze-thaw cycles prior to analysis in triplicate (mean ± S.D. shown). One vial was opened at each thaw cycle, and the other vial was not opened until 20 freeze-thaw cycles were completed. There was no significant difference between the fresh and incubated samples (Repeated measures ANOVA, p = 0.66).

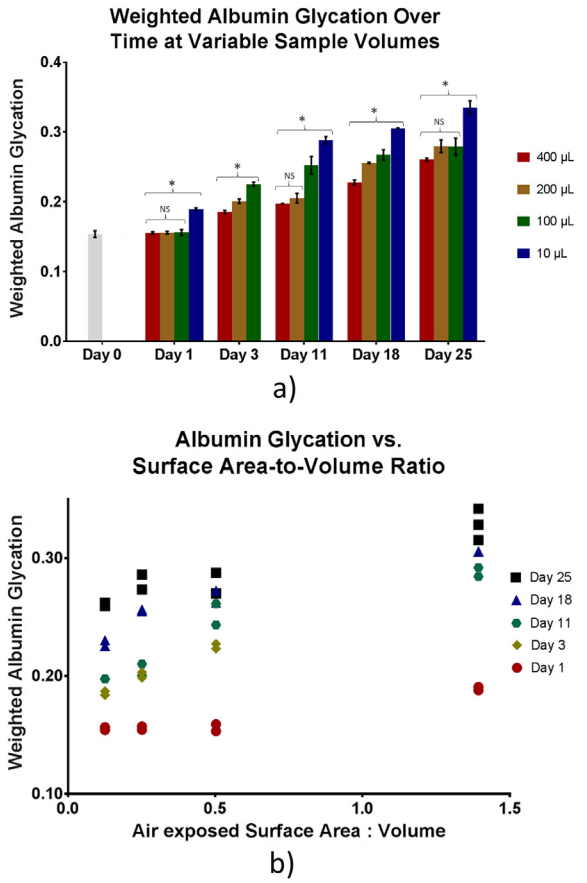

Fig. 5. Effect of surface area-to-volume ratio on the *ex vivo* increase in plasma albumin glycation at room temperature. WAG in plasma samples of different volumes over time at room temperature **(A),** each of which had a different surface area-to-volume ratio **(B).** Bar graphs represent the average of duplicate analyses with the error bars representing the actual points. On day 1 there was a significant difference between the 10 μL sample and all other samples. On day 3 and 18 there was a significant difference between all volumes (i.e., surface area-to-volume ratios). For day 11, there was a significant difference between all volumes except 400 and 200 μL. On day 25 there was a significant difference between the 10 μL sample and all other sample volumes. (* Represents a significant difference between surface area-to-volume ratios as determined by ANOVA; $p < 0.05$ for all Holm-Sidak's pairwise comparisons. "NS" indicates that the bracketed samples were not significantly different.). Individual data points are shown in the scatter plot **(B).** In this plot, the sample with the highest sa/vol ratio corresponds to the lowest-volume sample (10 μL) shown in (A); accordingly, sa/vol ratios decrease (B) as volume increases (A). For all days sa/vol ratios were positively correlated with albumin glycation (Pearson Correlation, $r \geq 0.9$, $p < 0.05$).

time points, the samples incubated under oxygen exhibited higher levels of WAG than the samples incubated under nitrogen (Fig. 6). After propagating the error for all measurements [34] the average *difference* between all nitrogen and oxygen data points past time zero was found to be 0.022 ± 0.005 WAG units, which was significantly different from zero (Wilcoxon matched pairs signed-rank test; p = 0.016). As a control experiment, data were also collected from samples kept in air; these data points largely fell in between those of the nitrogen and oxygen data points (Supplemental Fig. 6).

4. Discussion

Although plasma and serum samples may appear to be frozen at −20 °C, they are not fully frozen or chemically inactive at this temperature. Previously, we have demonstrated that protein oxidation occurs slowly but inevitably when P/S samples are stored at −20 °C [9]. Herein we have shown that glycation of albumin in

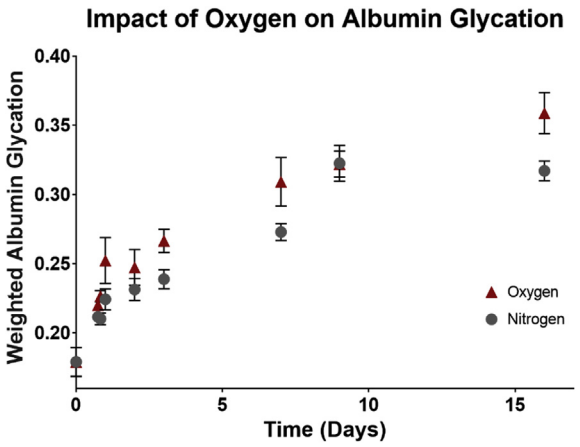

Fig. 6. Role of atmospheric oxygen on the *ex vivo* glycation of albumin in blood plasma. WAG in healthy-donor plasma kept under nitrogen or oxygen at room temperature (n = 3 aliquots measured per data point). The average difference between the oxygen and nitrogen series was significantly different from zero (Wilcoxon matched pairs signed-rank test; p = 0.016).

blood plasma of both healthy and diabetic donors increases substantially over time at temperatures at or above the known plasma freezing point of −30 °C [4–7]. It is important to note that when samples were stored at −80 °C there was no significant alteration of WAG *ex vivo*. This is consistent with the stability of protein glycation observed in other studies [35–37].

Samples shown in Fig. 3 were collected fresh, at which point WAG in the healthy donor was 17% and WAG in the pcT2D donor was 28%. This difference in initial WAG values was expected based on the higher average concentration of blood glucose that is observed in T2D patients [38,39] and reported above. Both samples experienced an *ex vivo* increase in albumin glycation: Over a time period of 268 days at −20 °C, the sample from the healthy donor increased to 34% WAG (a relative increase of 100%, Fig. 3A). After 229 days, the sample from the pcT2D donor increased to 66% (a relative increase of 136%, Fig. 3A). The large difference in the absolute increase in percent WAG can be explained by the fact that fasting plasma glucose in the pcT2D patient was nearly 2.5 times the concentration found in the healthy donor.

In 2013, Watano et al. showed that GA in diabetics experienced a *relative* increase of 17% after 6 months and 73% after 12 months when serum was stored at −20 °C [37]. The albumin-specific enzymatic method employed by Watano et al. accounts for multiple glycation events per albumin molecule and is thus similar to our WAG metric. Differences in the relative increases in glycated albumin between our study and the Watano study might be accounted for by the concentration of free glucose in the samples when drawn, but these were not reported by Watano et al. [37]. Notably, Watano et al. observed the greatest increases in GA between 6 and 12 months of their 12-month study, but the greatest increases in WAG in our study occurred within the first 6 months (Fig. 3A). The reason for this discrepancy in the kinetic profiles is unknown.

Though numerous groups have evaluated the stability of protein glycation *ex vivo* [35–37], the mechanism(s) behind observed increases in protein glycation in P/S have not been elucidated. *Ex vivo* increases in protein glycation are likely due to either of two mechanisms: First, the chemical reaction between glucose and protein may not be at chemical equilibrium *in vivo*. This mechanism is most likely to be relevant to proteins with short half-lives. Albumin has a half-life of about 20 days [40]. At 37 °C the rate constant for the overall rate-limiting formation of the Amadori product from the Schiff based intermediate is 0.026 hr^{-1} [41]. As a first-order reaction, the half-life for the Schiff base intermediate is given by the equation $t_{1/2} = \ln 2/k$ and is therefore 26.7 h. Thus, the reaction can effectively be considered to be at equilibrium within one week—a time period well within the ~20 day half-life of albumin. Considered in conjunction with the fact that blood samples were drawn from fasting subjects, it is rather unlikely that the increase in albumin glycation observed *ex vivo* is simply due to chemical equilibration.

A second possible mechanism behind the *ex vivo* increase in albumin glycation is “autoxidative” glycation [20,21]. When P/S is exposed to air it takes on a dissolved oxygen concentration of approximately 0.25 mM [2,3]—a concentration far higher than that found *in vivo* where oxygen in the blood is carried by hemoglobin inside of red blood cells. To begin to ascertain the role of oxidative processes in the *ex vivo* glycation of albumin in P/S, samples were incubated for several days under atmospheres of nitrogen or oxygen (Fig. 6). Starting at 18 h glycation was, on average, elevated under an oxygen atmosphere relative to a nitrogen atmosphere. These results suggest that the mechanism underlying the *ex vivo* increase in albumin glycation involves molecular oxygen—a finding that is further substantiated by the facts that (1) increased plasma surface area-to-volume ratios enhanced the rate of *ex vivo* glycation (Fig. 5) and (2) plasma storage at −20 °C in vials with holes in them resulted in significantly greater *ex vivo* glycation than when the same sample was stored in vials without holes (Supplemental Fig. 5).

The precise molecular mechanism of autoxidative glycation is not yet clear. In 1987 Wolff and colleagues first reported roles for transition metals and oxygen in the autoxidation of glucose [21]—a phenomenon that accounted for up to 45% of the covalent attachment of glucose to bovine serum albumin when the two components were incubated together in air. They postulated that the two-electron oxidation of glucose mediated by transition metals and molecular oxygen produced glucosone, an α-dicarbonyl derivative of glucose that readily reacts with protein amino groups. However, in 1995 Wells-Knecht and colleagues demonstrated that only arabinose and glyoxal (but not glucosone) are produced during the autoxidation of glucose, neither of which would result in a 162 Da mass shift upon attachment to a protein molecule. Moreover, they demonstrated that glucosone is not even an intermediate in the formation of arabinose or glyoxal during the oxidative degradation of glucose [33]. This being the case, the only way to reconcile the findings of these two groups with our findings that a 162-Da molecule attaches to albumin during exposure of P/S to thawed conditions is to propose that the enediol radical anion of glucose that has been postulated to form upon initial one-electron oxidation [21] reacts directly with albumin (rather than a molecule of oxygen or a transition metal atom, which facilitates the second-electron oxidation of glucose in the Wolff mechanism [21]). Considering that the total concentration of free transition metals in P/S is in the low micromolar range and that the concentration of oxygen in P/S is approximately 0.25 mM [2,3] but the concentration of albumin is higher—at approximately 0.65 mM [42] (putting the total number of reactive amino-group equivalents with albumin in the millimolar range), this mechanism for the oxidative *ex vivo* glycation of albumin in P/S may be viable—though it has yet to be proven.

In conclusion, this study demonstrated that albumin glycation increases substantially *ex vivo* when fasting P/S specimens from either healthy subjects or diabetics are stored at −20 °C or otherwise subjected to temperatures above their melting point of −30 °C. The mass spectra employed to quantify albumin glycation revealed the presence of multiple glycation events per albumin molecule (Supplemental Fig. 3), affording the opportunity to account for these events in a metric referred to here as weighted

albumin glycation. Furthermore, experiments conducted under atmospheres of nitrogen vs. oxygen in conjunction with studies on the impact of P/S surface area-to-volume ratio strongly suggested a role for autoxidative processes during the *ex vivo* glycation of albumin in human P/S.

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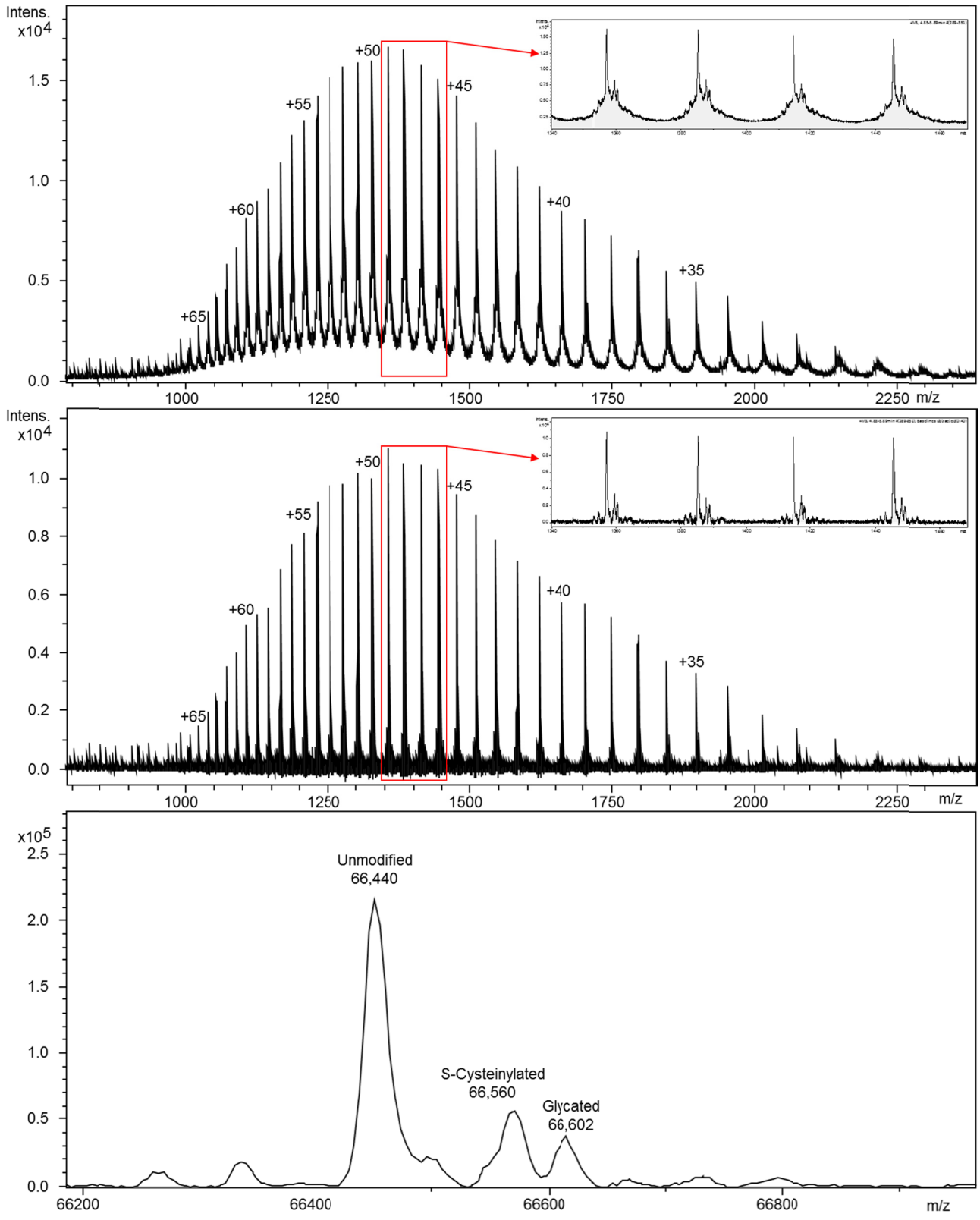
Appendix A. Supplementary data

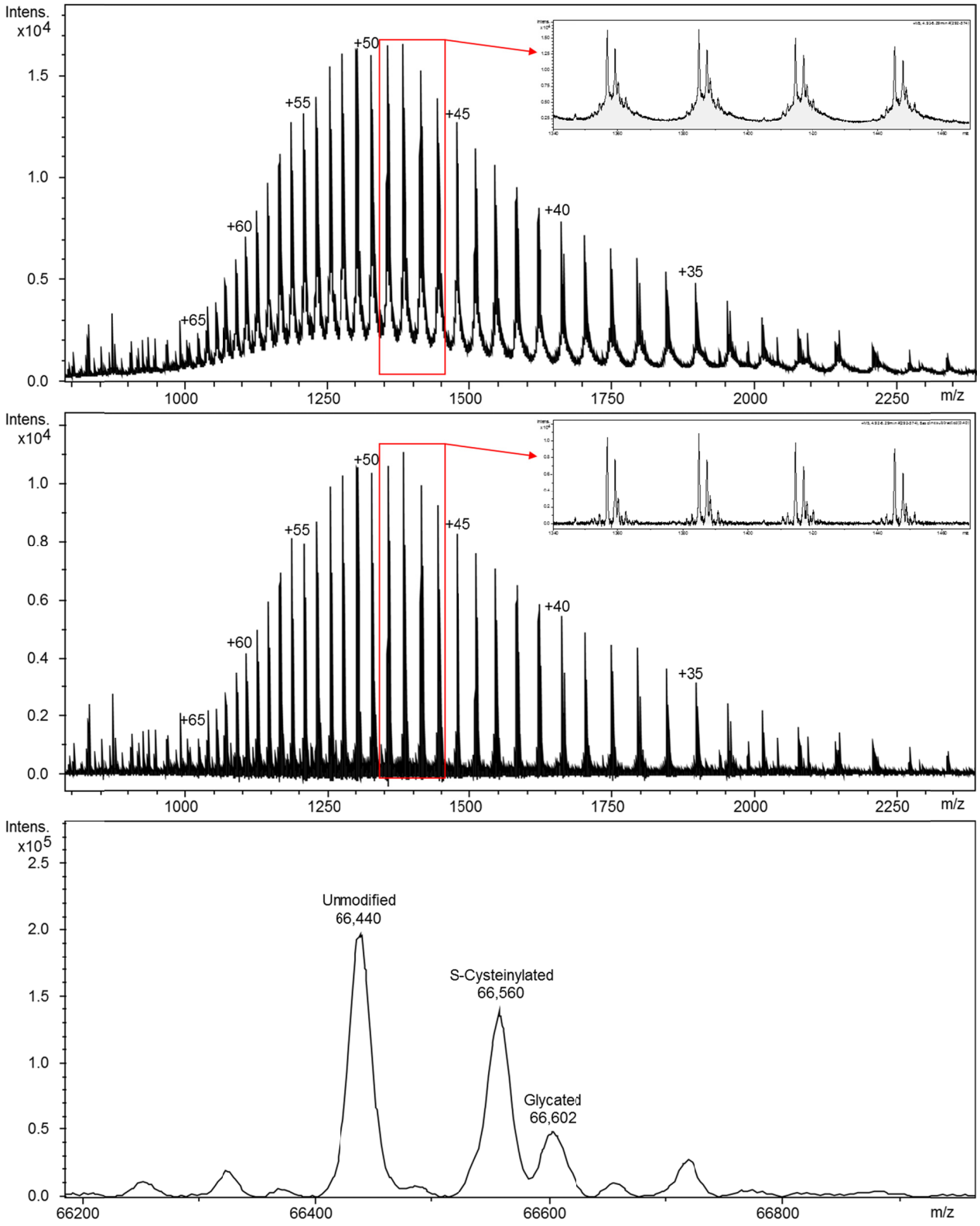
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.abb.2017.07.004.

References

[1] C. Exley, M.J. Mold, The binding, transport and fate of aluminium in biological cells, J. Trace Elem. Med. Biol. 30 (2015) 90–95.
[2] L.H. Gevantman, Solubility of selected gases in water, in: W.M. Haynes (Ed.), CRC Handbook of Chemistry and Physics, CRC Press/Taylor and Francis, Boca Raton, FL, 2015, pp. 149–152 pp. Section 5.
[3] G.R. Buettner, In the absence of catalytic metals ascorbate does not autoxidize at pH 7: ascorbate as a test for catalytic metals, J. Biochem. Biophys. Methods 16 (1) (1988) 27–40.
[4] A. Farrugia, R. Hill, S. Douglas, K. Karabagias, A. Kleinig, Factor VIII/von Willebrand factor levels in plasma frozen to-30 degrees C in air or halogenated hydrocarbons, Thromb. Res. 68 (1) (1992) 97–102.
[5] A.P. MacKenzie, First and second order transitions during the freezing and thawing of source plasma (human), in: American Institute of Chemical Engineers Symposium: Processing and Fractionation of Blood Plasma, Philadelphia, 1980, pp. 8–12. June.
[6] M.I. Bravo, S. Grancha, J.I. Jorquera, Effect of Temperature on Plasma Freezing under Industrial Conditions, Pharmeuropa Scientific Notes, vol. 2006, 2006, pp. 31–35, 1.
[7] Human Plasma for Fractionation, Monograph 0853. Ph. Eur. Suppl 5.3 Strasbourg, Council of Europe, France, 2005.
[8] R.V. McIntosh, A.J. Dickson, D. Smith, P.R. Foster, Freezing and thawing of plasma, in: C.T.S. Sibinga, P.C. Das, H.T. Meryman (Eds.), Cryopreservation and Low Temperature Biology, Kluwer Academic Publishers, Norwell, MA, 1990, pp. 11–24.
[9] C.R. Borges, D.S. Rehder, S. Jensen, M.R. Schaab, N.D. Sherma, H. Yassine, B. Nikolova, C. Breburda, Elevated Plasma Albumin and Apolipoprotein A-I Oxidation under suboptimal specimen storage conditions, Mol. Cell Proteomics 13 (7) (2014) 1890–1899.
[10] A.M. van den Besselaar, E. Witteveen, F.J. van der Meer, Long-term stability of frozen pooled plasmas stored at -70 degrees C, -40 degrees C, and -20 degrees C for prothrombin time and International Normalized Ratio (INR) assessment, Thromb. Res. 131 (4) (2013) 349–351.
[11] F. Betsou, E. Gunter, J. Clements, Y. DeSouza, K.A. Goddard, F. Guadagni, W. Yan, A. Skubitz, S. Somiari, T. Yeadon, R. Chuaqui, Identification of evidence-based biospecimen quality-control tools: a report of the international society for biological and environmental repositories (ISBER) biospecimen science working group, J. Mol. Diagn 15 (1) (2013) 3–16.
[12] S. Pasella, A. Baralla, E. Canu, S. Pinna, J. Vaupel, M. Deiana, C. Franceschi, G. Baggio, A. Zinellu, S. Sotgia, G. Castaldo, C. Carru, L. Deiana, Pre-analytical stability of the plasma proteomes based on the storage temperature, Proteome Sci. 11 (1) (2013) 10.
[13] G. Anton, R. Wilson, Z.H. Yu, C. Prehn, S. Zukunft, J. Adamski, M. Heier, C. Meisinger, W. Romisch-Margl, R. Wang-Sattler, K. Hveem, B. Wolfenbuttel, A. Peters, G. Kastenmuller, M. Waldenberger, Pre-analytical sample quality: metabolite ratios as an intrinsic marker for prolonged room temperature exposure of serum samples, PLoS One 10 (3) (2015) e0121495.
[14] G. Sozzi, L. Roz, D. Conte, L. Mariani, F. Andriani, P. Verderio, U. Pastorino, Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays, J. Natl. Cancer Inst. 97 (24) (2005) 1848–1850.
[15] C.R. Borges, J.W. Jeffs, E.P. Kapuruge, Impact of artifactual, ex vivo oxidation on biochemical research, in: M. Hepel, S. Andreescu (Eds.), Oxidative Stress: Diagnostics and Therapy, Vol. 2, American Chemical Society, Washington, DC, 2015, pp. 375–413.
[16] M. Brownlee, Advanced protein glycosylation in diabetes and aging, Annu. Rev. Med. 46 (223) (1995) 34.
[17] J. Baker, P. Metcalf, R. Scragg, R. Johnson, Fructosamine Test-Plus, a modified fructosamine assay evaluated, Clin. Chem. 37 (4) (1991) 552–556.
[18] T. Kouzuma, T. Usami, M. Yamakoshi, M. Takahashi, S. Imamura, An enzymatic method for the measurement of glycated albumin in biological samples, Clin. Chim. Acta 324 (1–2) (2002) 61–71.
[19] T. Kouzuma, Y. Uemastu, T. Usami, S. Imamura, Study of glycated amino acid elimination reaction for an improved enzymatic glycated albumin measurement method, Clin. Chim. Acta 346 (2) (2004) 135–143.
[20] J.V. Hunt, R.T. Dean, S.P. Wolff, Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing, Biochem. J. 256 (1) (1988) 205–212.
[21] S.P. Wolff, R.T. Dean, Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes, Biochem. J. 245 (1) (1987) 243–250.
[22] R.R. Little, C.L. Rohlfing, A.L. Tennill, S. Connolly, S. Hanson, Effects of sample storage conditions on glycated hemoglobin measurement: evaluation of five different high performance liquid chromatography methods, Diabetes Technol. Ther. 9 (1) (2007) 36–42.
[23] C. Constanti, J.M. Simo, J. Joven, J. Camps, Serum fructosamine concentration in patients with nephrotic syndrome and with cirrhosis of the liver: the influence of hypoalbuminaemia and hypergammaglobulinaemia, Ann. Clin. Biochem. 29 (Pt 4) (1992) 437–442.
[24] P. Koskinen, K. Irjala, Stability of serum fructosamine during storage, Clin. Chem. 34 (12) (1988) 2545–2546.
[25] R. Beck, M. Steffes, D. Xing, K. Ruedy, N. Mauras, D.M. Wilson, C. Kollman, G. Diabetes Research in Children Network Study, the interrelationships of glycemic control measures: HbA1c, glycated albumin, fructosamine, 1,5-anhydroglucitrol, and continuous glucose monitoring, Pediatr. Diabetes 12 (8) (2011) 690–695.
[26] M.P. Morais, J.D. Mackay, S.K. Bhamra, J.G. Buchanan, T.D. James, J.S. Fossey, J.M. van den Elsen, Analysis of protein glycation using phenylboronate acrylamide gel electrophoresis, Proteomics 10 (1) (2010) 48–58.
[27] S. Mashiba, K. Uchida, S. Okuda, S. Tomita, Measurement of glycated albumin by the nitroblue tetrazolium colorimetric method, Clin. Chim. Acta 212 (1–2) (1992) 3–15.
[28] E. Dozio, N. Di Gaetano, P. Findeisen, M.M. Corsi Romanelli, Glycated albumin: from biochemistry and laboratory medicine to clinical practice, Endocrine 55 (3) (2017) 682–690.
[29] B.M. Murphy, S. Swarts, B.M. Mueller, P. van der Geer, M.C. Manning, M.I. Fitchmun, Protein instability following transport or storage on dry ice, Nat. Methods 10 (4) (2013) 278–279.
[30] Downloaded from, The Early Detection Research Network (EDRN) Standard Operating Procedure (SOP) for Collection of EDTA Plasma, August 2013, http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/plasma-sop.pdf.
[31] Downloaded from, The Early Detection Research Network (EDRN) Standard Operating Procedure (SOP) for Collection of Serum, August 2013, http://edrn.nci.nih.gov/resources/standard-operating-procedures/standard-operating-procedures/serum-sop.pdf.
[32] D. Bar-Or, K.D. Heyborne, R. Bar-Or, L.T. Rael, J.V. Winkler, D. Navot, Cysteinylation of maternal plasma albumin and its association with intrauterine growth restriction, Prenat. Diag 25 (3) (2005) 245–249.
[33] K.J. Wells-Knecht, D.V. Zyzak, J.E. Litchfield, S.R. Thorpe, J.W. Baynes, Mechanism of autoxidative glycosylation: identification of glyoxal and arabinose as intermediates in the autoxidative modification of proteins by glucose, Biochemistry 34 (11) (1995) 3702–3709.
[34] D.A. Skoog, D.M. West, F.J. Holler, S.R. Crouch, Fundamentals of Analytical Chemistry, ninth ed., Cengage - Brooks/Cole, Belmont, CA, 2014 (Chapter 6)-Random errors in chemical analysis.
[35] O. Rolandsson, S.L. Marklund, M. Norberg, A. Agren, E. Hagg, Hemoglobin A1c can be analyzed in blood kept frozen at -80 degrees C and is not commonly affected by hemolysis in the general population, Metabolism 53 (11) (2004) 1496–1499.
[36] W. Jones, J. Scott, S. Leary, F. Stratton, S. Smith, R. Jones, A. Day, A. Ness, A.S. Team, Stability of whole blood at -70 degrees C for measurement of hemoglobin A(1c) in healthy individuals, Clin. Chem. 50 (2) (2004) 2460–2461.
[37] T. Watano, K. Sasaki, K. Omoto, M. Kawano, Stability of stored samples for assays of glycated albumin, Diabetes Res. Clin. Pract. 101 (1) (2013) e1–2.
[38] J. Anguizola, R. Matsuda, O.S. Barnaby, K.S. Hoy, C. Wa, E. DeBolt, M. Koke, D.S. Hage, Review: glycation of human serum albumin, Clin. Chim. Acta 425 (2013) 64–76.
[39] H.V. Roohk, A.R. Zaidi, A review of glycated albumin as an intermediate glycation index for controlling diabetes, J. Diabetes Sci. Technol. 2 (6) (2008) 1114–1121.
[40] T. Peters Jr., Serum albumin, Adv. Protein Chem. 37 (1985) 161–245.
[41] J.W. Baynes, S.R. Thorpe, M.H. Murtiashaw, Nonenzymatic glucosylation of lysine residues in albumin, Methods Enzym. 106 (1984) 88–98.
[42] United States Center for Disease Control and Prevention, National Health and Nutrition Examination Survey (NHANES), 2006.



Supplementary Figure 1



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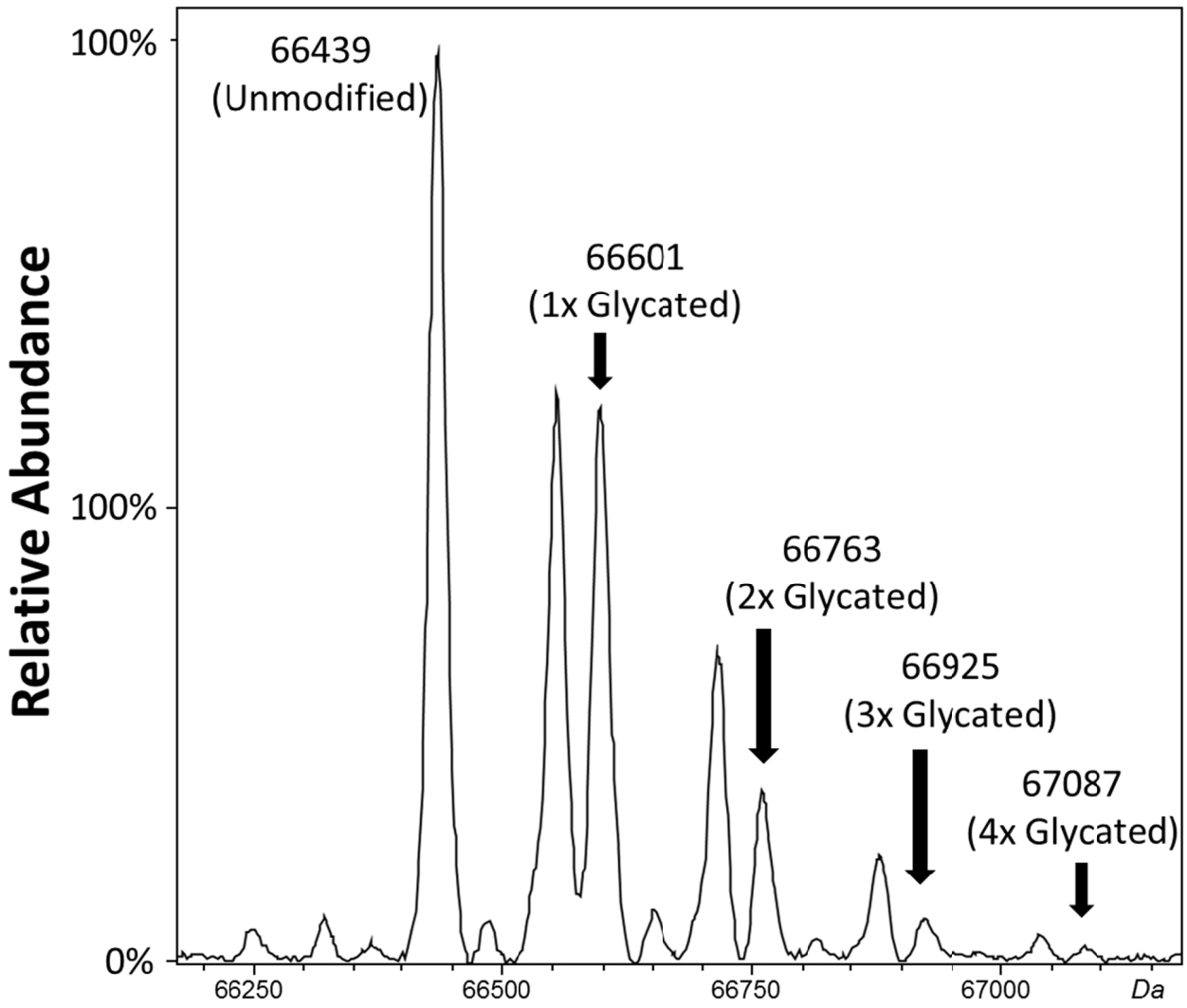

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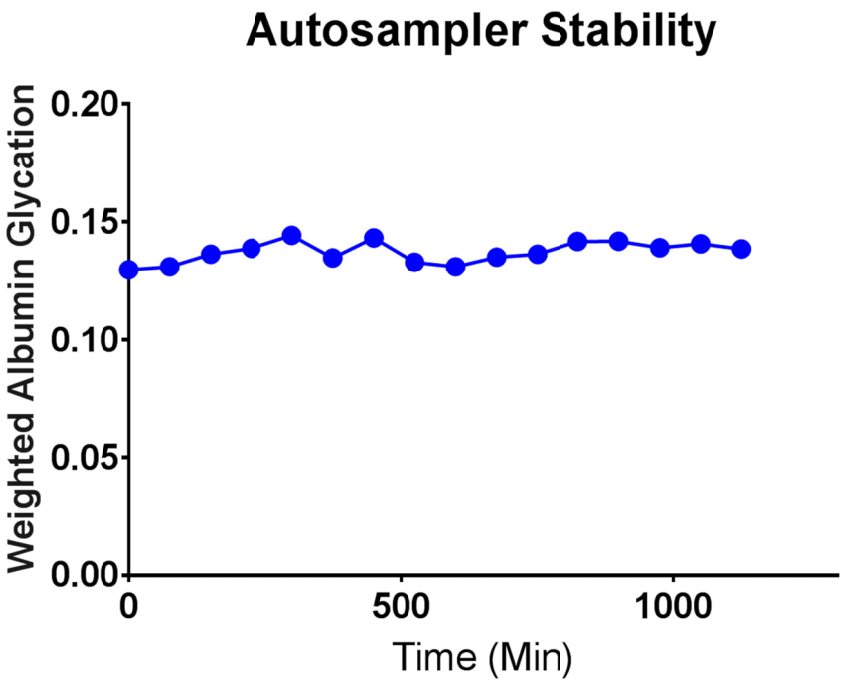

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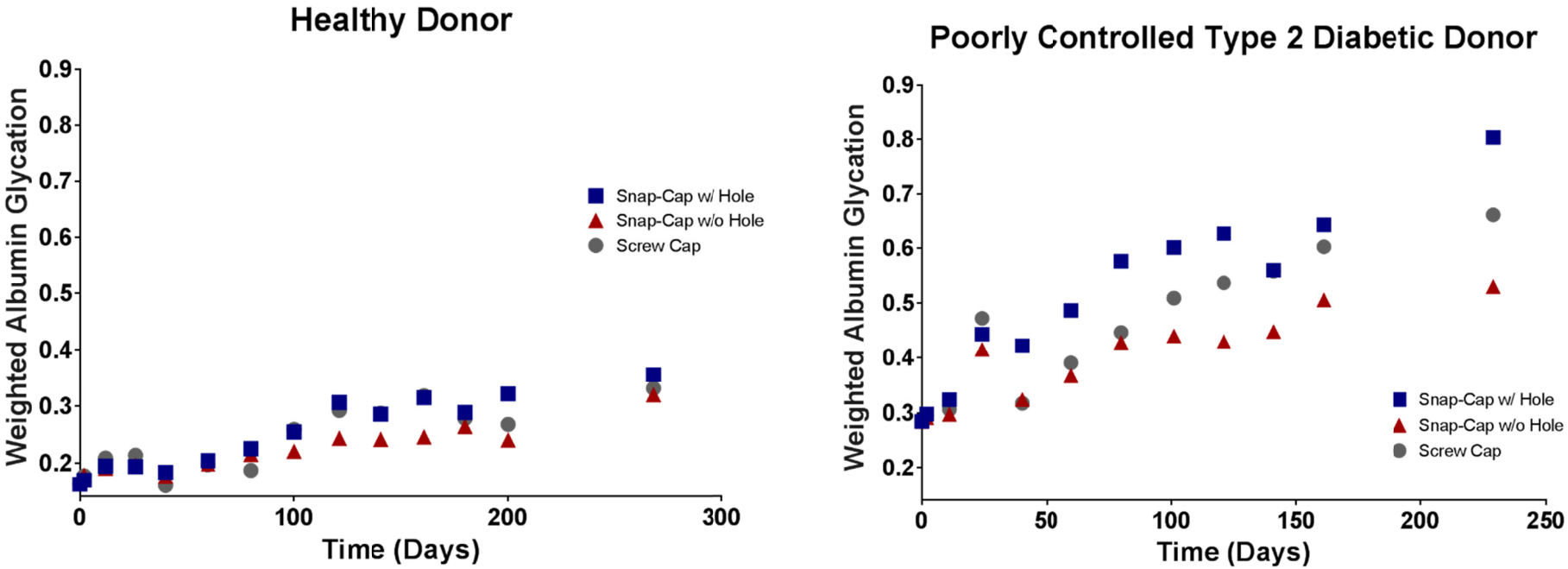
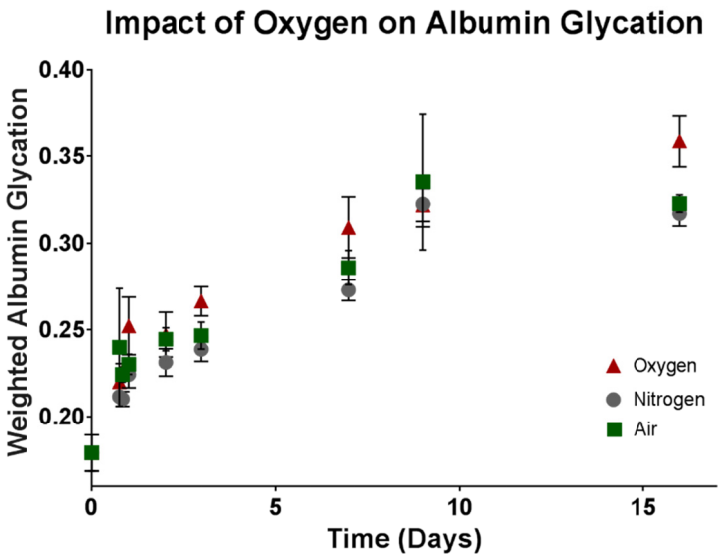


Supplementary Figure 5



Supplementary Figure 6