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Isothermal Vitrification Methodology Development for Non-cryogenic Storage of Archival Human Sera

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Abstract

Biorepositories worldwide collect human serum samples and store them for future research. Currently, hundreds of biorepositories across the world store human serum samples in refrigerators, freezers, or liquid nitrogen without following any specific cryopreservation protocol. This method of storage is both expensive and potentially detrimental to the biospecimens. To decrease both cost of storage and the freeze/thaw stresses, we explored the feasibility of storing archival human serum samples at non-cryogenic temperatures using isothermal vitrification. When biospecimens are vitrified, biochemical reactions can be stopped, the specimen ceases to degrade, and macromolecules can be stabilized without requiring cryogenic storage. In this study, 0.2, 0.4, or 0.8 M trehalose; 0, 0.005 or 0.01 M dextran; and 0 or 10% (v/v) glycerol was added to human serum samples. The samples were either dried diffusively as sessile droplets or desiccated under vacuum after they are adsorbed onto glass microfiber filters. The glass transition temperatures (T_g) of the desiccated samples were measured by temperature-ramp Fourier Transform Infrared (FTIR) spectroscopy. Sera samples vitrified at 4 ± 2 °C when 0.8 M trehalose and 0.01 M dextran were added and the samples were vacuum dried for two hours. Western immunoblotting showed that vitrified serum proteins were minimally degraded when stored for up to one month at 4 °C. About 80% of all proteins were recovered after storage at 4 °C on glass microfiber filters, and recovery did not decrease with storage time. These results demonstrated the feasibility of long-term storage of vitrified serum at hypothermic (and non-cryogenic) temperatures.

Keywords

Isothermal vitrification; serum; non-cryogenic storage; desiccation; glass transition temperature; stabilization; trehalose

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Introduction

Currently, millions of serum biospecimens are being stored in biorepositories across the nation while tens of thousands of new biospecimens are added to the pool daily. These biospecimens are archived for future research, mainly for proteinaceous biomarker discovery and verification (e.g. for diagnostic, therapeutic, and epidemiologic outcomes) [7; 28; 58]. The success of biomarker research not only depends upon the availability of the tools (proteomic, peptidomic, lipidomic and metabolomic technologies) to extract information, but also on the availability of “high quality” biospecimens [51]. However, in most biorepositories, biospecimens are stored by freezing without following any preservation protocol; the samples are directly placed in -20 , -40 or -80 °C freezers, in the absence of any cryoprotectant, where they experience very slow cooling ($1-2$ °C/min). It is well known that these conditions impose very harsh chemical and physical stresses on macromolecules [46], altering their characteristics (structure and activity), often irreversibly [36; 41; 44].

Most macromolecules are damaged during cooling, freezing, cryogenic storage, and thawing due to:

- a. *Temperature effects*: The native structure of proteins may be destabilized at low temperatures, making the denatured (unfolded) state thermodynamically favorable (i.e. cold-denaturation) [19];
- b. *Osmotic/dehydration stress*: Low water chemical activity in the freeze-concentrate decreases the free energy of the denatured state, making it thermodynamically preferred [26];
- c. *pH shift*: Cooling and freezing induce significant changes in ionic solubility and pH [53], affecting protein structure;
- d. *Protein-protein and protein-solute interactions*: These interactions are enhanced due to freeze-concentration induced crowding, hydrophobic interactions and changes in free energy [27];
- e. *Protein-ice interactions*: Many proteins are adsorbed onto the ice surface, where they aggregate and denature [15]. In addition, proteins experience mechanical compression during ice growth and re-crystallization as well as hydrostatic pressure increase due to volume change during freezing (water volume increases by 10% during freezing), which cause further denaturation, and aggregation. Frozen biospecimens undergo further damage during thawing, especially if it occurs slowly.

Most macromolecules in serum are affected by freezing and storage at cryogenic temperatures, some more extensively than others [25; 36; 41; 44; 46]. Many of the most promising proteinaceous cancer biomarkers were shown to be very susceptible to freeze/thaw and frozen state storage [16; 30]. For example, in sera from cancer patients, the levels of albumin, fibrinogen, and C3a decrease significantly in correlation with the length of storage; C3a was considered as a breast cancer biomarker until it was discovered to be very sensitive to storage conditions [20; 37]. Freezing lactate dehydrogenase (LDH), under any condition, causes damage that is completely irreversible and detrimental [22]; LDH is a biomarker used clinically and is currently being evaluated as testicular cancer biomarker [55; 56]. Metalloproteinase-9 (MMP-9) starts to degrade at -80 °C, dropping by 65% in activity within two years of storage [49]. More recent studies that utilize highly sensitive techniques such as LC-MALDI-TOF and MALDI-FT-ICR-MS have reported significant effects of repeated freeze/thaw on the proteome [8; 48]. Specifically, the MMP family (MMP-1, MMP-7, MMP-9, MMP-13) [49] and a related family, ADAMs (a disintegrin and

metalloprotease) that are considered to be diagnostic and prognostic biomarkers in all major cancers including breast, pancreas, lung, bladder, colorectal, ovarian, prostate and brain [50]; TIMP-1 (tissue inhibitors of metalloproteinases) [29]; polymeric proteins such as transthyretin (that forms fibrils leading to amyloidosis) [48] as well as glycoproteins [36], and even small molecules such as folate, and thyroid hormones [25; 44] are shown to be very susceptible to freeze/thaw.

Moreover, most of the studies conducted to date on biomarker storage stability were confined to mass spectrometry (MS) analysis, which is adequate for the measurement of the relative amounts of peptides and proteins or their absence/presence. However, MS does not provide information on the changes in the secondary and tertiary structure, state of denaturation or aggregation (or in enzymatic biomarkers, the activity) of proteinaceous biomarkers [23]. Therefore, the detrimental effect of frozen state storage is potentially even more significant than that reported to date in the literature and it is plausible that frozen state storage causes certain important biomarker information in the stored biofluid biospecimens to be lost forever. This is a major issue for the archival biospecimens currently stored in the biorepositories for future research. The importance of this problem becomes painfully obvious given that quality of the biospecimens is the “Achilles’ Heel” of the technologies being developed for personalized medicine.

For vitrification (i.e. to form a “glass,” which is a very viscous fluid), glass-forming osmolytes (such as sugars, polyols and organic polymers), which are also presumed to be lyoprotectants, are added to the liquid to be vitrified and the sample is desiccated, freeze-concentrated or freeze-dried (lyophilized) [41; 46]. In isothermal vitrification, the samples are desiccated at room temperature, without freezing. In the glassy state, due to the high viscosity of the fluid, the biochemical reactions are halted, degradation of the specimen is stopped, and macromolecules are stabilized in their native states [1; 2; 3; 47]. Therefore, it is hypothesized that isothermal vitrification can store the biospecimens stably at non-cryogenic temperatures, without exposing the proteinaceous biomarkers to the freeze/thaw stresses or to frozen-state storage damage and therefore could substantially increase the quality of the stored biospecimens.

Other significant benefits of vitrified state stabilization/storage are:

- i. It eliminates the freezing requirement and the resulting damage caused by ice;
- ii. It enables non-cryogenic storage (e.g. 4 °C), and even potentially room temperature storage (20–24 °C), eliminating the requirement for the freezer-farms, which is a very expensive investment, costly to maintain and operate, and is prone to failures (power shortage, break-down, human error, etc.) that could easily destroy the banked biospecimens by exposing them to repeated freeze/thaw cycles;
- iii. Water is removed from the biospecimen, reducing its volume and thus the storage space requirement per specimen;
- iv. Elimination of the cold-chain requirement and reduction of sample volume and weight reduces shipment costs significantly. Elimination of the requirement of shipping biospecimens on dry ice alone has a significant economical benefit;
- v. It does not require toxic chemicals (such as DMSO used in cell preservation, which is environmentally and economically costly to dispose); and
- vi. Some biobanks receive large aliquots of samples, which require thawing, re-aliquoting, and refreezing [51]. Vitrified samples can easily be divided into smaller pieces by cutting or breaking, while most frozen specimens require thawing of the whole specimen for sampling.

The long-term goal of our research is to eliminate the requirement for frozen state storage and develop the techniques to store archival serum biospecimens at room temperature using isothermal vitrification technology with special emphasis on stabilization of proteinaceous biomarkers. As a first step in this direction in this research, we translate the information generated in a parallel field, pharmaceuticals (where stability of therapeutic macromolecules and drugs are of utmost importance) [46], to biospecimens stabilization and explore the feasibility of storing archival human serum biospecimens in a vitrified state using isothermal vitrification.

Materials and Methods

Serum Specimen Preparation and Desiccation

Trehalose (TRE), a 342 Da disaccharide known for its cryo-/lyoprotectant capability and relatively high glass transition temperature (95 °C) [4; 33; 39; 54], was selected for this study as the main osmolyte to protect the serum proteins during drying. Glycerol (GL) was used, in addition to TRE, for its cryoprotective characteristics [13; 34; 35]. GL is also known to work synergistically with TRE to dampen the high frequency molecular vibrations in a glass [11; 12], enhancing the stability of pharmaceutical formulations. Finally, 35 – 45 kDa dextran (DEX) was used to increase the glass transition temperature, T_g , of the formulation [31; 42], facilitating isothermal vitrification. High purity (99 %) anhydrous TRE was purchased from Pfanstiehl (Ferro Pfanstiehl Laboratories, Waukegan, IL), while all other chemicals were purchased from Sigma (Sigma-Aldrich Corp., St. Louis, MO).

Human serum samples were collected from volunteers following a University of Minnesota Institutional Review Board (IRB) approved protocol (Study Number: 1011E92892). Serum samples (0.15 μ L) containing additives (0.2, 0.4 or 0.8 M TRE; 0 or 10 % v/v GL; and 0, 0.005 or 0.01 M DEX) were placed on IR transparent CaF_2 windows and were either: 1) diffusively dried at an environment of less than 2% relative humidity (RH) for one day, one week, or one month or 2) vacuum dried at 250 torr at room temperature for 30 minutes, 1, 2 or 4 hours.

Measurement of the Homogeneity of the Serum Samples Desiccated as Sessile Droplets

To determine post-drying sample homogeneity, serum samples containing lyoprotectants were dried on CaF_2 windows (see above) and transferred to the IR microscope and IR spectra were collected across the entire diameter of the sample at 150 μ m increments. The second derivative of the IR spectra was calculated and the peak intensities at 1150 cm^{-1} (ν -CO vibrations of TRE), 1550 cm^{-1} (Amide II), and 2150 cm^{-1} (water combination peak) were determined. Ratios of the peak intensities were used to calculate the relative concentrations of the different components (TRE, protein and water, respectively) in the desiccated sample. Residual water content of the bulk sample was determined by gravimetric analysis using an HB43 Moisture Analyzer (Mettler Toledo International Inc.). In the gravimetric analysis 300 μ L of dried sample was heated at 100 °C for 45 minutes to remove all residual water (to obtain the dry weight of the sample).

Measurement of the Glass Transition Temperature (T_g)

After lyoprotectant doped serum samples were dried for pre-determined time periods, their glass transition temperatures (T_g) were measured. T_g is defined as the temperature at which the viscosity of the sample exceeds 10^{13} Pa•s and thus, for all practical purposes, the sample is considered to have reached the glassy state [4]. Temperature-ramp Fourier Transform Infrared (FTIR) spectroscopy [40; 47] was conducted while the samples were cooled from 20 °C to –80 °C at a cooling rate of 2 °C /min using a cryostage (Linkam Scientific Instruments, UK) placed under a Nicolet Continuum FTIR microscope (Thermo Electron

Corporation, LLC., Waltham, MA). The second derivative of the IR spectra (in the range of $940 - 7000 \text{ cm}^{-1}$) was calculated and the position of both the protein Amide II (1550 cm^{-1}) peak and the 1150 cm^{-1} peak (corresponding to the ν -CO vibrations of the glycosidic bond of TRE) were recorded. T_g was determined from the slope in the temperature-dependent peak shift kinetics following protocols previously published elsewhere [40; 57]. The Amide II peak was examined to determine protein mobility while the 1150 cm^{-1} peak was examined to determine mobility of TRE. This was done to probe for potential decoupling of the mobility of the ingredients in the sample [47]. Droplet images were collected using the Nicolet Continuum FTIR microscope (Thermo Electron Corporation, LLC., Waltham, MA).

Desiccation and Elution of Serum Samples after Adsorption on Filter

In selected experiments, $100 \mu\text{L}$ of serum samples prepared following the experimental procedures presented above were absorbed onto 4 cm diameter glass microfiber filter paper (Whatman LTD, England) and vacuum dried at 250 torr for 4 hours. This was done to increase the homogeneity of the sample (see Results). After storage in a sealed flask (to stop further drying of the sample) at 4°C for specified time periods (from one day up to one month), the filter papers containing the desiccated serum samples were cut into pieces and placed into a 0.5 ml centrifuge tube that contained a hole in the bottom. Four hundred microliters of ultrapure water was added to rehydrate the desiccated serum sample. The 0.5 ml centrifuge tube was placed in a larger tube (1.5 ml) and centrifuged twice at 65g for 45 seconds to elute the proteins. The protein concentration in the eluate was measured using the bicinchoninic acid (BCA) protein assay (Pierce Protein Research Products, Rockford, IL) following the manufacturer's instructions. Protein recovery from the filter was calculated as a percentage of the protein concentration of the serum sample before adsorption onto the filter.

Protein Stability Analysis

Polyacrylamide Gel Electrophoresis (SDS-PAGE)—To determine whether serum proteins were degraded, aggregated, or depleted following isothermal vitrification, storage and rehydration, the samples were denatured and separated by molecular size by SDS-PAGE as previously described [5]. Briefly, $1.0 \mu\text{g}$ of serum protein from each treatment/timepoint was denatured in Tris-glycine sample buffer (0.625 M Tris, 10 % (w/v) glycerol, 0.05 % bromophenol blue, 1 % (w/v) SDS) with 1 % β -mercaptoethanol and separated on a precast 4–20 % gradient gel (BioRad, Hercules, CA) in Tris-glycine buffer (25 mM Tris, 192 mM glycine) with 0.1 % SDS.

Silver stain—Gels were stained with silver to visualize total protein as previously described [5].

Western blots—Western blots were used for the detection of individual serum proteins as previously described [5]. Briefly, proteins from the SDS-PAGE gels were transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare Biosciences, Pittsburgh, PA) in Tris-glycine buffer with 12.5 % (v/v) methanol. PVDF membranes were blocked overnight in phosphate buffered saline (PBS) containing 5 % nonfat dry milk and 0.05% Tween-20, then incubated in a 1:10,000 dilution of rabbit anti-human haptoglobin antibody (ab85846, Abcam, Cambridge, MA) in blocking solution. Following 3 washes in PBS containing 0.05% Tween-20, the PVDF membranes were incubated in a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase labeled secondary antibody (Pierce). The membrane was developed with West Femto chemiluminescence substrate (Pierce) and exposed to Kodak $\times 500$ film (Midwest Scientific, Valley Park, MO). The membrane was then stripped in buffer comprised of 0.2 M glycine, 0.1 % SDS, and 1 % Tween 20, pH 2.2 and then incubated with a 1:250,000 dilution of rabbit anti-human serum albumin (Advanced

Targeting Systems, San Diego, CA), followed by a secondary antibody incubation and development with the chemiluminescence substrate as described above for haptoglobin.

Results

Spatial Homogeneity of the Samples Desiccated as Sessile Droplets

Desiccation may result in heterogeneous drying of the specimen [3; 9; 10; 47]. This implies that the specimen is not uniformly at the same thermodynamic state. As a result, some regions within the specimen may degrade during storage much faster. This could be a major problem for the storage stability of the specimens [47]. To determine the spatial homogeneity of the dried serum samples, spatial mapping was conducted using scanning FTIR spectroscopy at $150 \times 150 \mu\text{m}$ resolution. Figure 1 shows a representative IR spectrum of human serum containing 0.2 M TRE that was vacuum dried for 4 hours. The ratio of the intensity of the Amide II peak located at 1550 cm^{-1} to the peak associated with the $\nu\text{-CO}$ vibrations of the glycosidic bond of trehalose (1150 cm^{-1} peak) was used to calculate the spatial distribution of the protein to TRE ratio in the desiccated sample following established protocols [47]. Spatial distribution of the water to protein ratio, on the other hand, was calculated using the water combination peak located at 2150 cm^{-1} and the Amide II protein peak. Figure 2 shows an image of the desiccated sessile droplet (left panels) and a graphical representation of the peak ratios measured at $150 \mu\text{m}$ increments across the droplet (protein to TRE ratio, center panels; water to protein ratio, right panels). Serum samples containing 0.2 M TRE had a $23 \pm 6.3 \%$ increase in protein content at the periphery of the sample (Figure 2A) as compared to the center of the droplet, while samples containing 0.4 M TRE had a $29 \pm 7.3 \%$ increase at the periphery (Figure 2B). This was caused by ring formation in the samples during drying due to peripheral adhesion and Marangoni instability [47]. Spatial homogeneity of the desiccated samples was increased by adding 10 % GL, which acts as a lubricant and a plasticizer [12]; decreasing accumulation at the periphery down to $2.75 \pm 1.6\%$ (Figure 2B,D). In contrast, the addition of 0.005M DEX to 0.4 M TRE increased the protein content at the periphery of the droplet by $43 \pm 5.5\%$ as compared to samples containing GL (Figure 2E).

In accord with the previous results from our group [47], we also observed increased cracking in desiccated sessile droplet samples containing only TRE upon drying as compared to the samples containing GL (Figure 2, left panels). Hydration levels of the proteins in the desiccated sera sample (represented by the water to protein ratio) also varied considerably among different samples (3rd column in Figure 2). Hydration of the proteins in the samples containing only TRE was also higher at the periphery potentially due to high affinity of TRE to water and the enhanced accumulation of TRE in the periphery. Addition of GL potentially dampened this affinity (due to the presence of more hydrogen bonding sites) and enabled more uniform distribution of protein hydration within the desiccated samples. The largest heterogeneity was seen in the samples containing dextran. This was expected since DEX, which is a sugar polymer, increases the viscosity of the liquids quite significantly. Residual water contents of the samples were found by gravimetric analysis. Samples containing GL had the lowest residual water content at $21.9 \pm 3.1\%$, while samples containing DEX had the highest residual water content at $34.5 \pm 5.4\%$. Samples containing only TRE as a lyoprotectant had $27.1 \pm 4.4\%$ residual water content.

Glass Transition Temperatures (T_g) of the Desiccated Samples

The glass transition temperature (T_g) of a sample is used as a measure of its stability at a given storage temperature (T_s). If $T_g > T_s$, it is presumed that the sample can be stored stably at T_s for a long period of time [21]. Note that the T_g of a sample depends on its composition and water content, as well as the glass-forming characteristics of the cryo-/

lyoprotectant agents added to the sample. Furthermore, as shown in the previous section, the desiccated samples are intrinsically heterogeneous. As the T_g of the samples reported here is a bulk value, the molecular mobility within the sample may vary significantly. Therefore, when evaluating the stability of any sample for storage after isothermal vitrification, its T_g and homogeneity should be considered together.

To determine whether the sugar and the protein vitrification kinetics are coupled we have calculated the T_g of the desiccated samples using two different spectral peaks; the Amide II band relating to the N-H bending vibrations and the C-N stretch vibrations of the protein molecules (1550 cm^{-1} , Figure 3A and 3C) and the ν -CO vibrations of the glycosidic bond of trehalose (1150 cm^{-1} peak, Figure 3B and 3D) [57]. The mobility of a macromolecule in a solution is correlated to its size and in certain extremes decoupling of the lyoprotectant and protein dynamics may be observed. This implies that even though the lyoprotectant reaches its percolation threshold and vitrifies, the proteins (especially small peptides) may still have sufficiently high conformational mobility. The obvious outcome is that the degradation rate of the sample might be much higher than that predicted by the bulk T_g measured based on the mobility of the lyoprotectant agent, and the sample degrades during storage. To accommodate for the potential decoupling (of the lyoprotectant and protein mobilities), in practice, vitrified samples are stored at a significantly lower temperatures than their T_g [24]. Our results show no significant difference between the T_g values calculated using the lyoprotectant spectra and the protein spectra ($p = 0.64$); indicating that in the compositions used decoupling was not a significant concern.

To evaluate the effect of different lyoprotective compounds on serum vitrification, T_g 's were measured for serum doped with 0.2 M, 0.4 M and 0.8 M TRE with and without 10% GL (Figure 3A and 3B), and 0.4 M and 0.8 M TRE with 0.005 M DEX and 0.01 M DEX (Figure 3C and 3D). Samples were dried at ambient temperature for 30 minutes to 4 hours. In general when TRE was held constant, T_g of the desiccated sera samples increased with desiccation time. Since GL is a known plasticizer (with a low T_g), its presence in the sample caused a decrease in T_g (Figure 3A and 3B). Addition of DEX, on the other hand, increased the T_g of the samples. DEX is a sugar polymer with a higher T_g than TRE and therefore, it increases the T_g of the solution even when it is used at low concentrations [3]; For example, in the current study, addition of 0.005 M or 0.01 M dextran resulted in a increase in T_g (Figure 3C and 3D).

In a preliminary experiment, serum containing 0.2 M, 0.4 M, or 0.8 M TRE with or without 10% (v/v) GL was dried at <2% RH at ambient temperatures and atmospheric pressure for one day, one week, and one month (Figure 4). T_g values were then calculated using Amide II peak. T_g of the serum samples containing 0.8 M trehalose dried for one month at <2% RH and ambient temperature reached 35 °C, suggesting that sera doped with a specific lyoprotectant cocktail can indeed be vitrified [3] and stably stored at room temperature without requiring cryogenic storage. However, drying the samples for a month at room temperature is not practical as significant protein degradation (and even crystallization) is likely to occur over the drying time period.

To accelerate drying time, we exposed the samples to lower vapor pressure by applying vacuum (Figure 3A–D). Similar to drying at atmospheric pressure, T_g 's increased with drying time, especially when the sera was doped with TRE only, or contained TRE + DEX. The presence of GL dampened the effect of drying time on T_g , particularly when the T_g was calculated using the Amide II peak (Figure 3A). Results showed that samples containing 0.8 M TRE reached a point to be stably stored in a standard refrigerator (~4 °C) after vacuum drying for only 4 hours. These samples had the same T_g values as the samples dried for one week at standard atmospheric pressure ($p = 1.00$). The effect of GL on T_g also was

significant in vacuum dried samples. When T_g 's of the samples only differing in their GL content were compared, it was seen that the T_g 's were uniformly lower in the presence of GL. Conversely, increasing the DEX concentration increased the T_g of the sample (Figure 3A–D).

Retention and Elution of Serum Proteins from Glass Fiber Filters

The results presented above showed that even though significantly high T_g values (4 °C) could be reached with serum samples relatively rapidly (within 2 hours) (Figure 3), homogeneity of the serum samples desiccated in the form of sessile droplets could still be an issue (Figure 2). Even though the degree of heterogeneity could be decreased by adding GL to the sample, the inevitable end result was a significant decrease in T_g . To minimize the heterogeneity of the samples during desiccation, in the second part of this study an alternative approach was utilized. This approach included using a standard glass fiber filter to adsorb the serum samples already doped with a lyoprotectant solution. It was expected that the mesoporous structure of the filter paper would induce a capillary force on the serum uniformly adsorbing it, and allowing it to be homogeneously desiccated.

One hundred microliters of serum with and without stabilizing additives (0.8 M TRE, 10% (v/v) GL, or 0.01 M DEX) was vacuum dried after being adsorbed onto glass microfiber filters at ambient temperatures for 4 hours and stored for 24 hours, 3 days, 7 days and 1 month at 4°C. After storage, the dried serum samples were rehydrated, eluted, and the protein concentration and the volume of eluate recovered were measured as described in the Materials and Methods section. Recovery of serum proteins (from the filter) ranged from 50–80% (Figure 5). Effect of additives on protein recovery was analyzed using linear regression models taking into account replicates and adjusting for time and additive by time interaction. All p-values were adjusted for multiple comparisons using a Bonferroni correction. Statistical analysis showed that 0.8 M TRE + GL sample had significantly lower protein recovery than either the untreated ($p = 0.0151$) or the 0.8 M TRE + DEX ($p = 0.0065$) sample.

Regardless of the length of time that the samples were kept in storage, those serum samples containing DEX always had the highest protein recovery, whereas the serum samples containing GL had the lowest protein recovery (Figure 5). The average volume of liquid recovered from the filters was approximately 85% of the volume of water used to elute the serum proteins, regardless of the additives and the centrifugation process used. These results suggested that some serum proteins remained entrapped in the glass microfiber filters together with the residual water (~15%) that could not be recovered.

Alteration of Specific Serum Proteins Following Vitrification and Storage

We next examined the composition and stability of the serum proteins recovered from filters by separating the proteins by SDS-PAGE and silver staining (Figure 6). Silver stained gels showed uniform protein elution with no specific proteins remaining in the glass microfibers (Figure 6A). As a more sensitive method of monitoring the stability of serum proteins, we examined the vitrified and eluted serum by Western blot analysis for human serum albumin and haptoglobin, both high abundant serum proteins (Figure 6B, C). The Western blots were probed with polyclonal antibodies, which recognize multiple epitopes on the proteins, in order to maximize the detection of degradation products. Full length, unprocessed human haptoglobin is a 42.7 kDa protein, which is proteolytically processed into alpha and beta subunits of 27.0 and 15.6 kDa, respectively. The antibody used recognizes the unprocessed protein and the haptoglobin alpha subunit. Haptoglobin did not appear to be degraded in eluted serum for any of the treatments or time points (Figure 6B). Similarly, no degradation of serum albumin was observed (Figure 6C). However, when 10% (v/v) GL was present in

serum containing 0.8 M TRE (i.e. serum proteins would not be in a vitrified state as shown in Figures 3A, B), both haptoglobin and albumin exhibited aggregation, as evidenced by the high molecular weight proteins visible on the Western blots (Figures 6B, C). Some minor aggregation of albumin was also observed in the untreated serum.

Discussion

Studies have shown that stability of protein structure during storage is directly correlated with post storage protein function and activity [45]. One critical parameter for storage stability of vitrified samples is the glass transition temperature (T_g), which has to be higher than the storage temperature (T_s). Another critical parameter is the homogeneity of the samples since this plays a major role on the degradation of the sample independent of the biopreservation method used, whether it is freezing or drying [15; 47].

Our results showed that desiccated sample homogeneity increases with the addition of 10% (v/v) GL. GL also appears to reduce crack formation during desiccation of the sample. Crack formations results from skin formation [1; 2] and the failure of the brittle skin due to the drying-induced mechanical stresses. Presence of cracks in the desiccated sample is not desirable since cracks act as foci inducing crystallization due to excess free energy of the interface. Furthermore, cracking increases the surface of the sample that could increase the risk of contamination, and moisture adsorption during storage. Therefore, to ensure sample stability, it is necessary to both increase sample homogeneity and decrease crack formation while aiming for as high a T_g as possible. However, the presence of GL in the samples decreased T_g .

Our data suggests that it is possible to vitrify human serum samples and stably store them at room temperature (e.g. 0.8 M TRE containing samples dried for one month at 2% RH with a T_g of 35°C; see Figure 4). However, the lengthy time requirement for desiccation is likely to result in significant protein degradation before the sample vitrifies. We showed that this can be remedied by reducing the drying time requirement by using vacuum drying. Because of the accelerated drying kinetics induced by vacuum drying, higher T_g values can be reached in a shorter drying period. Our results showed that serum samples containing 0.8 M TRE + 0.01 M DEX after vacuum drying for 2 hours have high enough T_g values that they can be stored under non-cryogenic conditions at $T_s = 4^\circ\text{C}$. While storage at 4 °C still requires the use of a refrigerator, the storage conditions are improved over the current practice because this can both substantially reduce storage expenses and also eliminate the stresses induced by frozen state storage.

While the average volume of fluid eluted from the glass microfiber filters was 85%, the average protein recovery ranged from 50 to 80% (Figure 5). These results suggest that proteins are interacting with the glass microfibers of the filter to such an extent that they could not be completely eluted with water. Interestingly, silver stained gels (within the detection limit of the technique) showed that serum proteins were uniformly eluted from the filters implying no specific proteins remained in the glass microfibers. While it is beneficial that no specific proteins remained adsorbed to the glass microfibers, many low abundance proteins (including clinically important biomarkers) make up <1% of the total protein content of serum [18]. The detection limits of the SDS-PAGE method makes it impossible to reach a conclusion whether any important proteinaceous biomarkers remain entrapped in the filter. This is an area that requires ongoing exploration, specifically focusing on determination of the affinity of different serum proteins to different filter materials to find the best material to be used in future studies. If no such material can be found, or eluting 100% of the serum proteins require extensive processing of the sample (for example by using detergents or acids), then alternative methodology utilizing filter materials that can

dissolve in water can be used. Our group has been exploring this alternative in parallel ongoing studies.

Samples containing DEX resulted in the highest protein recovery; this may be a consequence of DEX being a relatively high molecular weight sugar polymer (40 kDa) such that DEX may specifically interact with the glass fibers by coating them and therefore creating a buffer region between the serum proteins and the filter material, therefore inhibiting adsorption. Conversely, serum samples containing GL resulting in the lowest protein recovery. GL has a much lower molecular weight (92 Da) than DEX and therefore may not be as capable of shielding proteins from interacting with the glass microfibers. Also the addition of GL to serum resulted in the aggregation of proteins (Figure 6); suggesting that the aggregation of protein within the filter pores might be responsible for reduced protein recovery.

Stresses induced during drying can also cause protein degradation and aggregation [14; 45; 46]. Therefore we explored degradation and aggregation in the vitrified serum after storage (at $T_s = 4^\circ\text{C}$) and elution to determine protein stability. Storage times ranged from 1 day to 1 month to obtain a more complete representation of protein stability over time. Western blots of specific serum proteins (haptoglobin and albumin) were chosen as general determinants of protein stability because these proteins appear in high concentration in normal human serum and they are easy to detect on stained gels. We found that serum containing 0.8 M TRE and 0.01 M DEX had the least amount of aggregation and degradation, and therefore this composition was determined to be the most stable (Figure 6). Samples containing no stabilizing sugars showed increased aggregation of albumin over time, which is to be expected considering these samples were devoid of lyoprotectants and therefore not vitrified at the 4°C storage temperature (data not shown). Finally, it was seen that addition of 10% (v/v) GL caused significant aggregation during vitrified state storage. While GL has been shown to increase homogeneity, which is critical for storage stability, there are several possible ways by which GL could decrease protein stability during storage. Firstly, our data shows that the addition of GL decreases T_g . Low T_g implies that the proteins may have enough conformational mobility during storage to undergo physical and chemical denaturation. Secondly, aggregation of proteins in the presence of GL could be a result of glycation due to the Maillard reaction [6] a chemical reaction between amino acids and a sugar. Maillard reaction has been shown to be extremely deleterious for proteins in the presence of increased concentrations of GL [38]. While the sample starts out with an initial GL concentration of 10% (v/v), this concentration increases drastically when the sample is desiccated, resulting in favorable conditions for the Maillard reaction.

Conclusion

Studies have shown that freeze/thaw, especially if repeated, leads to protein degradation [43; 52]. This could be deleterious for many cancer biomarkers, which have been shown to be extremely susceptible to stresses induced by freeze-thaw [17; 30; 32]. In fact, because of the known detrimental effects of freezing, frozen-state storage is rarely used for preservation of labile pharmaceutical proteins [46]. Because of biomarkers sensitivity to freeze-thaw stresses, a new method of stabilization and storage is necessary to preserve specifically the biospecimens that contain biomarkers important for cancer detection and research. Storage via isothermal vitrification may be a feasible alternative to frozen state storage. Our results showed that serum samples can be stored in the glassy state at 4°C when 0.8 M TRE is added and the sample is vacuum dried at ambient temperature for 4 hours. With the addition of 0.01 M DEX, drying time requirement may be reduced to 2 hours. Western blot analysis shows that samples stored for up to one month at 4°C appear to have neither aggregation nor degradation of proteins when 0.8 M TRE and 0.01 M DEX are present. However, when

stabilizing sugars are not added to the sample, aggregation of large proteins, such as albumin is inevitable.

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Figure 1.
Example of IR spectrum of human serum sample containing 0.2 M TRE. Sample was vacuum dried at 0% relative humidity for 4 hours at room temperature.

Figure 2.

Ratios of protein to TRE and protein to water as a function of location in 0.15 μL vacuum dried droplet of sera containing: (A) 0.2 M TRE; (B) 0.2 M TRE and 10% (v/v) GL; (C) 0.4 M TRE; (D) 0.4 M TRE and 10% (v/v) GL; and (E) 0.4 M TRE and 0.005 M DEX. All measurements were normalized with respect to the ratio of protein to TRE (left graphs) or protein to water (right graphs) measured at the center of the droplet. **Images:** Human sera dried as sessile droplets on the CaF_2 slides (note that droplets are approximately 600 μm in diameter).

Figure 3.

Glass transitional temperature (T_g) values for serum samples with additives. **(A)** T_g values calculated using the Amide II peak at 1550 cm^{-1} for dried serum containing 0.2 M, 0.4 M, or 0.8 M TRE with or without 10% (v/v) GL. Samples were vacuum dried at ambient temperature for 30 minutes (diamonds), 1 hour (squares), 2 hours (triangles), or 4 hours (circles). **(B)** Samples prepared in the same manner as **(A)**, but T_g values were calculated using the 1150 cm^{-1} peak of TRE. **(C)** T_g values were calculated using the Amide II peak at 1550 cm^{-1} for dried serum containing 0.4 M or 0.8 M TRE with 0.005 M or 0.01 M DEX. Samples were vacuum dried at ambient temperature for 30 min to 4 hours as in **(A)**. **(D)** Samples prepared in the same manner as **(C)**, but T_g values were calculated using the 1150 cm^{-1} peak of TRE. Mean of three experiments $\pm$ SEM.

Figure 4. T_g values calculated using Amide II peak, of dried serum containing 0.2 M, 0.4 M, or 0.8 M TRE with or without 10% (v/v) GL. Samples were dried at <2% RH at ambient temperatures and atmospheric pressure for one day (diamond), one week (square), and one month (triangle). Mean of three experiments ± SEM

Figure 5.

Percent recovery of serum proteins from glass fiber filters after vitrification, storage, and elution. One hundred μ l serum without additives or treated with 0.8 M TRE, 10% (v/v) GL, or 0.01 M DEX was dried on a glass microfiber filter and stored at 4°C for 24 hours, 3 days, 7 days or 1 month before the proteins were eluted in excess ultrapure water (see Materials and Methods). The total protein recovered from the filters was determined and is expressed as a percentage of the protein initially adsorbed onto the filters. No additives (white column), 0.8 M TRE (grey column), 0.8 M TRE and 10% (v/v) GL (striped column); 0.8 M TRE and 0.01 M DEX (black column). Mean of three experiments $\pm$ SEM.

Figure 6.

The stability of serum proteins after vitrification and elution from the microfiber filter. Serum without additives or treated with 0.8 M TRE, 10% (v/v) GLY, or 0.01 M DEX was dried on a glass microfiber filter and eluted in ultrapure water after storage for 24 hours, 3 days, 7 days, and 1 month at 4°C as in Figure 4. One μ g of each sample was run on a 4–20% SDS-PAGE and then: **(A)** silver stained for total protein; **(B)** transferred to a PVDF membrane and probed with a polyclonal antibody against haptoglobin. Full length, unprocessed human haptoglobin is a 42.7 kDa protein, which is proteolytically processed into alpha and beta subunits of 27.0 and 15.6 kDa, respectively. The antibody used recognizes the unprocessed protein and the haptoglobin alpha subunit. **(C)** **(B)** transferred to a PVDF membrane and probed with a polyclonal antibody to haptoglobin, stripped, and then probed with a polyclonal antibody against human serum albumin. The arrows point to haptoglobin bands, which were not completely removed by the stripping buffer.