Silk-based blood stabilization for diagnostics

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Advanced personalized medical diagnostics depend on the availability of high-quality biological samples. These are typically biofluids, such as blood, saliva, or urine; and their collection and storage is critical to obtain reliable results. Without proper temperature regulation, protein biomarkers in particular can degrade rapidly in blood samples, an effect that ultimately compromises the quality and reliability of laboratory tests. Here, we present the use of silk fibroin as a solid matrix to encapsulate blood analytes, protecting them from thermally induced damage that could be encountered during nonrefrigerated transportation or freeze–thaw cycles. Blood samples are recovered by simple dissolution of the silk matrix in water. This process is demonstrated to be compatible with a number of immunoassays and provides enhanced sample preservation in comparison with traditional air-drying paper approaches. Additional processing can remediate interactions with conformational structures of the silk protein to further enhance blood stabilization and recovery. This approach can provide expanded utility for remote collection of blood and other biospecimens empowering new modalities of temperature-independent remote diagnostics.

silk | blood | protein | stabilization | diagnostics

Blood contains a variety of proteins, enzymes, lipids, metabolites, and peptides, which can be interrogated as biomarkers for health screening, monitoring, and diagnostics. The integrity of these blood components and thus the quality of information attained from their analysis is determined by the storage conditions from sampling until analysis, or the so-called pre-analytical phase (1, 2). This phase often includes time-consuming processing steps and the requirement for a continuous cold storage. Without temperature regulation, blood-derived biospecimens degrade quickly, accounting for up to 67% of all laboratory testing errors (2, 3). Further, when blood-derived materials are frozen, decreases in thermodynamic free energy and unfavorable ice crystal–protein interactions (4) can occur during subsequent thawing (5, 6), which can further compromise analyte integrity as a gold-standard methodology.

As an alternative route, blood and blood derivatives can be dried via newer approaches such as isothermal vitrification (7), lyophilization (8), or on silica chips (9). Isothermal vitrification and lyophilization are inherently resource-intensive techniques and thus not suitable for field use. Silica chips are designed for enrichment of selected fractions of the low-molecular-weight serum proteome, but are not broadly protective at elevated temperature (10). An inexpensive alternative used since the 1960s are dried blood spots (DBS) (11), a paper card system which captures blood components among cellulose fibers as the water phase evaporates. These drying measures decrease sample weight by >90%, thus decreasing transport burden, and in theory can enhance long-term sample stability by decreasing water-dependent analyte degradation caused by hydrolysis and enzyme activity (12). Unfortunately, DBS stored in challenging environments (i.e., elevated temperatures and humidity) quickly lose their protective capacity (13, 14). Indeed, years of field research supported by the Centers for Disease Control has shown that DBS on-card drying approaches do not fully safeguard all blood analytes (15). Piloting DBS cards for the National Health and Nutrition Examination Survey revealed unreliable recovery of cholesterol markers, which was attributed to oxidative stress (16). Thus, although newborn screening programs have relied heavily on DBS (17), cold storage of DBS is still considered a requirement for use, and this fact has limited widespread adoption of dried sample preservation formats to solve worldwide cold chain issues.

A new class of biomaterials based on the silk fibroin protein is being considered to address a wide range of stabilization challenges, from labile enzymes (18) to antibodies (19). Silk fibroin (hereafter referred to as silk) is a high-molecular-weight amphiphilic protein (20). Silk is generated in an aqueous state and is thus readily miscible with both simple protein solutions as well as complex fluids such as whole blood (21). As a protein polymer, silk can be processed into a variety of mechanically robust formats including films (18) and gels (22). Self-assembly of silk into solid structures can be initiated through physicochemical factors (23) or by water removal (21, 24). Although these particular features are not specific to silk, we and others have examined similar excipients for use as stabilizing matrices such as albumin, collagen, gelatin, and sugars. Along with immunogenicity and manufacturing sustainability concerns with collagen and gelatin, materials formed from these biopolymers are not as mechanically robust as silk and have lower glass transition temperatures. Although both albumin and sugars are used frequently in freeze-dried pharmaceutical formulations, neither can form self-standing constructs. Taken together, the drawbacks of common excipients and the DBS technology explain the scarcity of a material ideal for stabilizing dried biospecimens in limited resource settings.

We hypothesized that the unique features of silk protein would offer stabilization of blood components to aid in remote sample retrieval and transport. To test this hypothesis, we identified silk solution processing characteristics that enhanced the solubility characteristics of final dried silk-containing matrices after storage,

Significance

Both research and clinical care often require blood to be collected away from the laboratory setting. Remote collection presents a logistical and financial challenge, as it requires continuous access to portable cold storage. Although there has been a thrust to develop means to bypass the cold chain, available technologies such as dried spots, remain ineffective. Specifically, these methods fail to stabilize labile protein biomarkers against thermal damage. Herein we describe an alternative silk matrix encapsulation technique that overcomes these limitations and can be deployed using a simple air-drying approach. Potential clinical and research applications of this technology are far-reaching, and could ultimately decrease hospital burdens, improve patient compliance to monitoring, and open up new testing options for currently underserved populations.

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which in turn enabled enhanced recovery of blood-based analytes. We then tested both the silk stabilizers alone as well as silk/blood mixtures under long-term storage conditions at elevated temperatures to simulate field conditions. In some circumstances, reduced recovery of analytes in the presence of silk was ameliorated by the addition of salts that reduced silk hydrogen bonding, thus extending known mechanisms of silk stabilization (19, 25, 26). These findings should translate to technologies for remote and refrigerator-free sample procurement, either as a surrogate method for a variety of outpatient blood collection needs (i.e., screening and chronic disease monitoring), or to serve researchers/clinicians who are far from centralized testing facilities. Examples of the latter case include large-scale epidemiologic studies, multicenter reference interval studies, remote pharmacological trials, and other nonclinical studies.

Results and Discussion

Silk Fibroin Stabilization Structures. To generate biospecimen-entrapped silk materials, silk solution was mixed with blood or plasma and air dried overnight. Samples were easily removed from their substrates following drying, and sectioned or punched for distribution to multiple tests by weight. Samples were aliquoted to 40-mg coupons and fully solubilized in PBS or in test diluent to reach concentrations in the working ranges indicated by the assays. The schematic shown in Fig. 1 depicts the simple assembly technique.

Silk Processing Determines Solubility, Recovery. Before conducting long-term stability studies, we identified silk purification techniques and materials preparation methods that would yield fully dissolvable silk matrix formats. The solubility of thin films generated by spreading the silk solution over a larger surface was compared with thick silk films generated by air drying after drop casting the solution (Fig. 2*A*). The effect of silk molecular mass on resolubility was examined for both geometries. As had been shown previously, lyophilized silk composed of high average molecular mass, between 160 and 400 kDa, leads to solubility issues that can be resolved by extending the duration of sericin extraction from silk to reduce the molecular mass, between 40 and 200 kDa (27). Thus, extracting the silk cocoons for increasing times (≥60 min) at the onset of the purification scheme effectively decreases the molecular mass of the protein (28), and thus resolves the resolubilization needs for the films derived from these purified silk solutions (Fig. 2*B*). Spreading thinner films also enhanced solubility over the thick film counterparts, due to rapid drying that prevents formation of insoluble β-sheet structures (29). When the sub-200-kDa silk thin film format was used, the solubility of all films after 12 mo of storage at various temperatures was retained. We observed that specific analyte recovery from plasma or whole blood entrapped in silk was positively correlated with both increased extraction times (thus, lower-molecular mass silk) and film thickness (Fig. 2*C*). The sub-200-kDa silk solutions formed matrices that provided maximum analyte recovery, independent of film thickness.

Stabilization and Protection from Freeze–Thaw Damage. After improving the solubility behavior of the silk films as the storage/stabilization matrix, a storage study was initiated at elevated temperatures

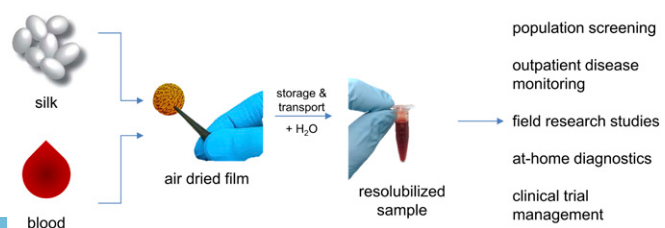


Fig. 1. Preparation schematic of air-dried silk-stabilizing matrices.

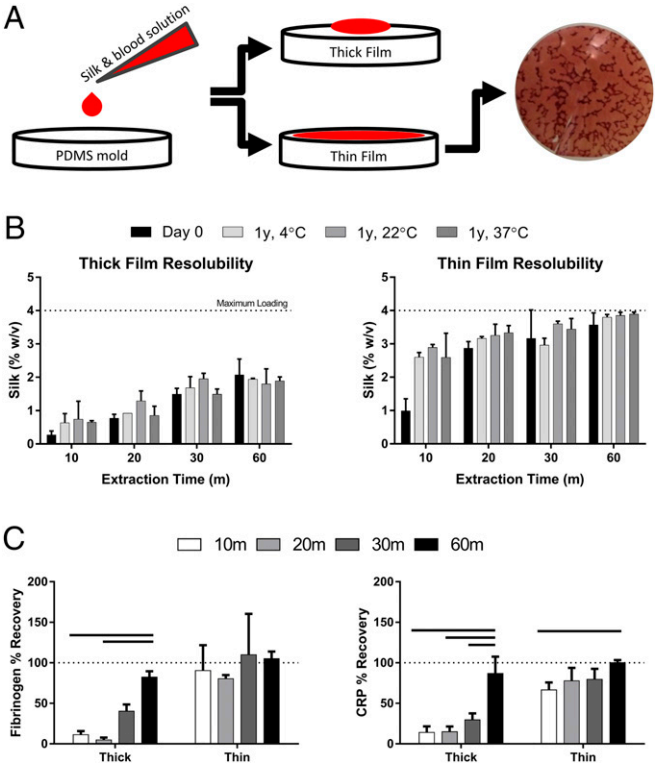


Fig. 2. Effect of silk molecular mass and film thickness on solubility and recovery. (*A*) Schematic of silk and blood solution casting into thick and thin films. (*B*) Resolubility of silk films after simulated aging conditions. Films were formed from solutions of varying molecular mass by control of fibroin extraction time. Dotted line represents maximum concentration of silk based on mass of coupon. (*C*) Recovery of 100 μL of plasma from silk matrices of varying silk extraction time and film thickness; % recovery is the silk film assay value normalized to the control plasma value on day 0; 100% indicated by the dotted line. Solid lines indicate significance at the $P < 0.05$ level.

to simulate field conditions and extended sample transport time frames. A Luminex immunoassay of different cardiovascular disease (CVD) markers was used as intended by the manufacturer (Table S1). The Luminex system examined six markers from donor blood samples from three separate donors after storage of samples for 30 d at 37 °C or room temperature (Fig. 3). Similar trends were found at day 30 as observed originally at day 0; the assay was sensitive to variations between donors and to the relative amounts of blood proteins isolated from whole blood vs. proteins isolated from purified plasma across all donors. Fibrinogen, soluble cell adhesion molecule (sVCAM-1), C-reactive protein (CRP), and serum amyloid P component (SAP) showed excellent agreement between theoretical loading levels (based on frozen plasma aliquots) and levels recovered from the blood and plasma coupons. In particular, CRP showed excellent fidelity between silk coupons and day 0 plasma measurements, despite donor levels ranging over 2 orders of magnitude. In the case of CRP and SAP, the two-way ANOVA resulted in significant sources of variation from donors but no significant differences between frozen plasma and either the blood or plasma coupons ($P < 0.05$). Taken together, these six different CVD markers demonstrated that the Luminex platform allowed discrimination of varying donor levels, independent of interactions with the silk matrix.

To generalize stability behavior across varying donors and time, the data in Fig. 3 was normalized to the day 0 plasma baseline values. Analytes showed complete, 100% recovery, except for the blood coupon measurements for CRP and haptoglobin, which is an intrinsic limitation of measuring these samples from unfractionated blood sources. The two-way ANOVA indicated significant variance

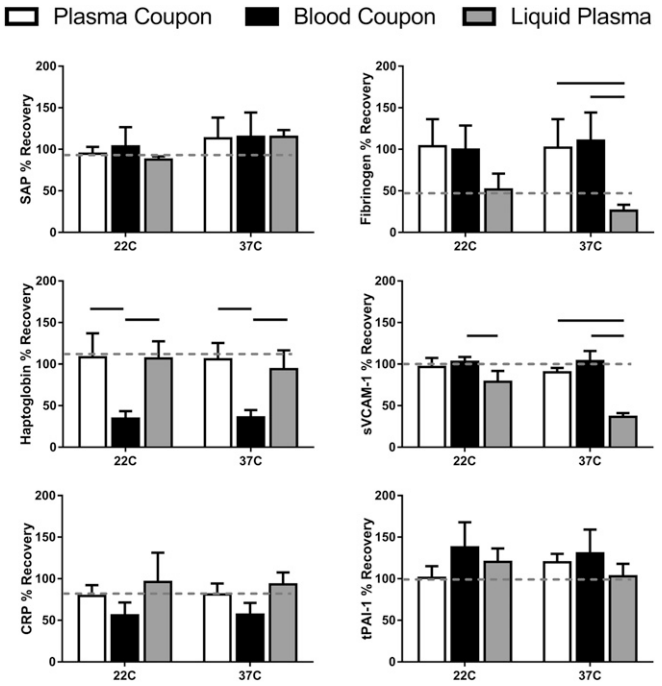


Fig. 3. Stability profiles of plasma in silk films (plasma coupons), blood in silk films (blood coupon), and liquid plasma from three donors after storage at 37 and 22 °C for 30 d. Here, % recovery is the plasma or silk film assay value obtained from 100 μL of encapsulated plasma normalized to control plasma value on day 0. The three donor percentage recovery values were averaged and the error bars represent SD. Dashed line represents liquid plasma levels stored at −20 °C for 30 d.

between the different storage formats. Specifically, fibrinogen and sVCAM-1 levels from silk-based films appeared insensitive to the storage conditions, whereas the respective liquid plasma levels decreased, significantly at 37 °C. Interestingly, the frozen plasma aliquot levels measured at day 30 (the dotted gray line on each graph in Fig. 3) did not always match the day 0 baseline values. CRP frozen plasma levels at day 30 were 80% of day 0 baseline, whereas fibrinogen levels were only 48% of the baseline, suggesting significant degradation of fibrinogen with the gold standard of freezing/thawing. This technique was avoided by using the silk film stabilization technique. Although designed for accelerated stability, these study conditions can mimic those encountered during some transportation environments, such as summer transit in hot courier trucks, in which dried systems free of cold storage may be exposed to elevated temperatures before arrival at testing laboratories (15).

Encapsulation Improves Stability over Dried Blood Spots. To evaluate silk against competing technologies, a storage study was performed examining the effect of time and temperature on the recovery of IgE from blood or serum between air-dried silk films and DBS. Total IgE levels are elevated in atopic individuals and antigen-specific IgEs are used for allergy identification (30). The technique has been described previously for dried blood spots (13). The results shown in Fig. 4 demonstrate excellent fidelity between serum and whole blood IgE in solution and IgE recovered from reconstituted films, and DBS eluent demonstrated artificially high readings for all three donors. This result arose from nonlinearity in concentration at different dilution levels in the ELISA. To fairly assess stability, the samples were normalized to the room temperature (22 °C) recovery sample, as is indicated for paper drying. Overall, the data show that there is a clear improvement of IgE stability using solid entrapment over maintaining the serum in the liquid state. Moreover, compared with the irregular output and overall diminished stability of IgE in DBS, IgE in silk was relatively stable at both temperature conditions for the duration of the study.

Targeting Assay Interferences. Variations on silk formulation and reconstitution media were used reconcile differences between assay values taken from fresh plasma samples and those derived from reconstituted plasma entrapped in silk. Previous studies demonstrated interferences from samples additives (such as buffers, protease inhibitors, or anticoagulants) can lead to artifacts in immunoassay results, which can be ameliorated through the use of additives such as chaotropes (31), or through alteration of sample matrix (32). Furthermore, chaotropes have been shown to destabilize drug-loaded silk micelles in solution, thus reducing shielding effects preventing the protein and drug from interacting (25). The effect of lithium bromide in the reconstitution media and titration of silk in the formulation was thus examined through the use of a 21-plex Luminex circulating cancer biomarker panel (Table S2). The plasma used in this study resulted in positive readings for 9 of the 21 available markers, as they were detectable at dilution levels recommended by the manufacturer. The ability to recover and stabilize these nine markers was assessed across two silk loadings (4% wt/vol and 1% wt/vol final concentration), and three reconstitution media (1 M LiBr in DiH_2O, 0.1 M LiBr in DiH_2O, DiH_2O).

We observed that, in most cases, complete recovery of analytes from air-dried silk matrices without additional intervention is possible (Fig. 5*A*). For instance, for the cases of total prostate-specific antigen (total PSA), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), leptin, carcinoembryonic antigen (CEA), prolactin, and stem cell factor (SCF), the typical film, formulated from 4% wt/vol silk reconstituted with DiH_2O, resulted in complete recovery after fabrication and also after storage at 22 and 45 °C for two weeks. These results were in contrast to the degradation patterns seen in liquid plasma or in plasma stored as dried spots (DPS; Fig. 5*A*). In other cases, however, interaction between the analyte and silk results in spurious readings (Fig. 5*B*).

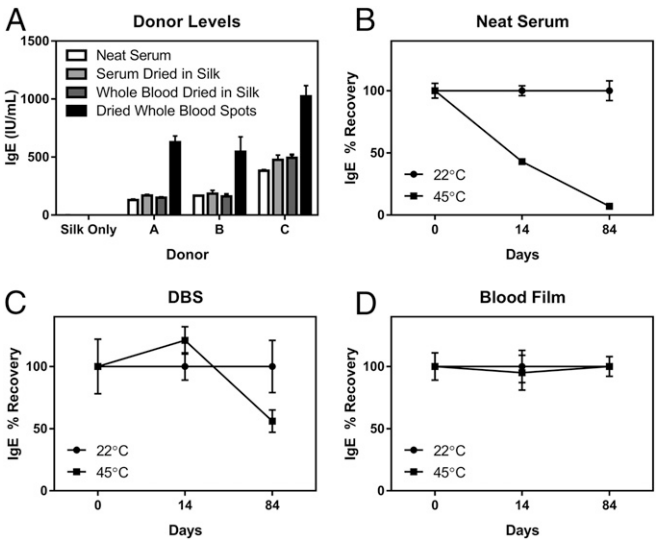
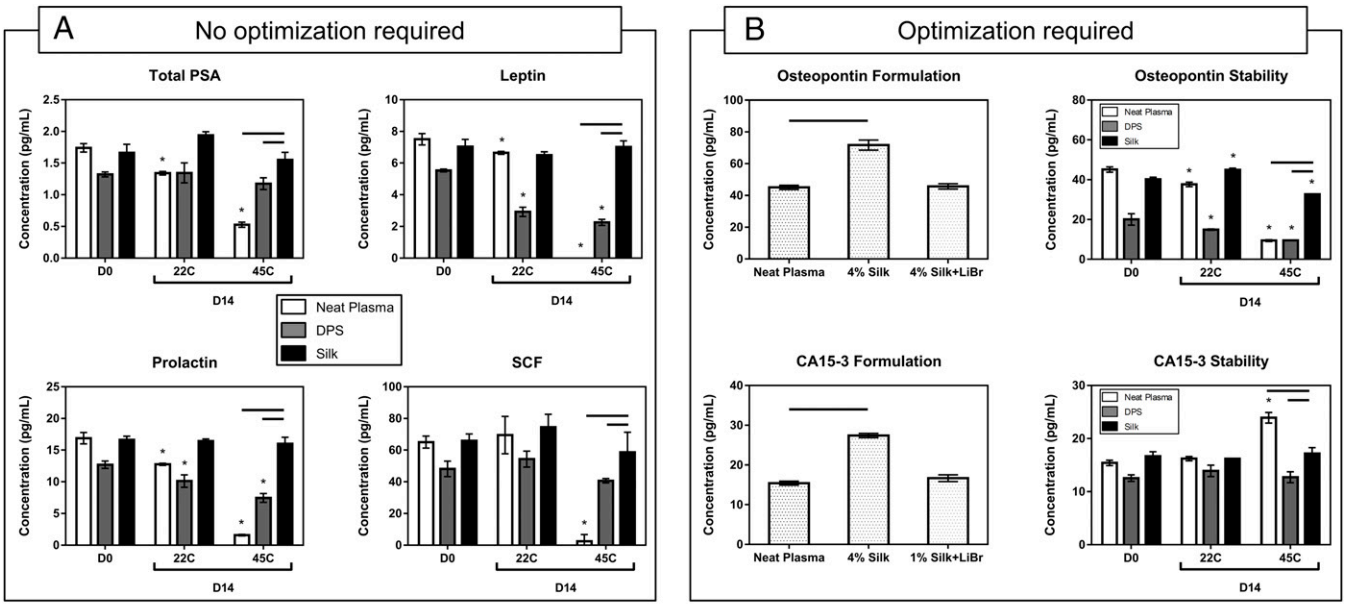


Fig. 4. Total IgE recovery and stability in silk compared with DBS. (*A*) Total IgE was assayed across three donor serum samples (*A*–*C*) via a commercial ELISA kit and compared with air-dried serum and silk solution, an air-dried whole blood and silk solution, and dried blood spots on Whatman 903 paper. Data represent of $n = 4$ replicates for each donor. (*B*–*D*) Liquid serum aliquots "neat serum," dried blood spots "DBS", and 50 μL of blood encapsulated in silk films "blood film" from the same three donors were subject to 22 or 45 °C storage over 84 d. Data are a pooled average ± SD of $n = 4$ replicates across three donors. Data were normalized to 22 °C recovery, the control condition.

For osteopontin (OPN), a film formulated from 4% wt/vol silk reconstituted with DiH_2O resulted in a falsely high reading that was ameliorated by reconstitution in 0.1 M lithium bromide (Fig. 5*B*). For cancer antigen 15–3 (CA15-3), a film formulated with 4% wt/vol silk and reconstituted with DiH_2O also resulted in falsely high readings that were resolved by the use of less silk (1% wt/vol) and the presence of 1 M lithium bromide (Fig. 5*B*). The formulation and reconstitution method that resulted in the reading closest to the neat plasma was evaluated after storage for 14 d and is shown in Fig. 5*C*. Addressing baseline recovery issues by including a chaotrope facilitated a direct comparison of the silk-laden samples to the baseline plasma controls and highlighted, for the majority of markers, the overall stability enhancements in head-to-head comparisons with DPS or temperature-matched plasma samples. Taken together, the results shown in Fig. 5 and elaborated in Table S4 demonstrate that the combination of 4% wt/vol silk without buffer in the casting formulation and 0.1 M LiBr in the reconstitution formulation proved adequate for most analytes.

Recovery of Biomarkers from Silk Is not Dependent on Patient Health Status. The experiments in Figs. 2–5 used biospecimens from randomly selected donors. To demonstrate the ability of the silk system to discriminate between healthy and diseased patients, plasma was obtained from a patient diagnosed with pancreatic cancer. These samples were assayed with and without the presence of silk (4 wt/vol% casting solution) following reconstitution with DiH_2O (Fig. 6). Both CA15-3 and osteopontin were excluded in the dataset due to the spurious signals that resulted from the formulation, as demonstrated in Fig. 5. The healthy patient was positive for 10 of the markers, and the diseased patient was positive for 11 of the markers. In both cases, air drying in the silk matrix resulted in high fidelity between the frozen neat plasma and the air-dried silk film (Fig. 6 and Table S5). The absolute differences between the liquid plasma control and (4 wt/vol%) silk-laden samples were similar to what was observed earlier (Fig. 5), although no attempt was made here to improve recovery via addition of salt or decreasing silk concentration.

Lyophilized Silk Powder as an Alternative to Silk Solution for Blood Stabilization. The studies shown in Figs. 2–6 used liquid silk solutions mixed with liquid biospecimens to generate air-dried films. Recently it was shown that freeze-dried silk powders are more temperature



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		Day 0				Day 14						
		Liquid Plasma	Silk Liquid	Silk Film	DPS	Liquid Plasma			Silk Film		DPS	
Storage Temp (°C)		-80	22	22	22	-80	22	45	22	45	22	45
Markers	Total PSA											
	CA15-3							↑				
	TRAIL											
	Leptin											
	CEA											
	sFas											
	Prolactin											
	SCF											
	OPN		↑									

Fig. 5. Eliminating assay interferences and uncovering silk stabilization mechanisms. (*A*) Examples of recovery and stability of biomarkers not requiring any changes to silk solution formulation. (*B*) Recovery of osteopontin from films dissolved in water with and without lithium bromide (*Left*) and stability of the films, control plasma, and DPS groups over time (*Right*). Recovery of cancer antigen 15–3 (CA15-3) from films generated from 4 wt/vol% silk solution and reconstituted in DiH_2O compared with silk films generated from 1 wt/vol% silk solution and reconstituted with lithium bromide (*Left*) and stability of the films, control plasma, and DPS groups over time (*Right*). For all graphs the donor recovery values were averaged and the error bars represent SD. Lines indicate significance between groups at the $P < 0.05$ level. Asterisks indicate groups that were significantly different from their respective day 0 readings at the $P < 0.05$ level. (*C*) Recovery and stability of biomarkers stored in different matrices for two weeks at –80 °C (liquid plasma only), 22 and 45 °C. Groups are color coded to indicate agreement with day 0 liquid plasma: green indicates no significant differences, yellow indicates levels significantly lower (but within 20% of day 0 levels), and red indicates both significant differences and levels >20% lower than day 0. In most cases stability issues led to decreased analyte levels; however, where applicable, the white arrows indicate levels significantly higher than day 0.

stable and durable than the silk solution from which they were generated (27) and can be readily dissolved for on-demand use. To probe the utility of this approach for purposes of blood stabilization, neutrophil gelatinase-associated lipocalin (NGAL), a urinary and plasma biomarker for acute kidney injury (33), was encapsulated in silk films formed from powders. NGAL was completely recovered from all three silk formats: solutions, films from solutions, and films from powders (Fig. 7*B*). We also examined the recovery of NGAL using the same Luminex panel evaluated in Fig. 5. Again, the integrity of the biomarkers recovered from the silk powder-generated film was maintained through the air-drying process regardless of abundance in plasma (Fig. 7*B*).

Conclusions

Here we used a simple method to encapsulate and transport small blood samples for long-term ambient storage and subsequent on-demand recovery and laboratory analysis. Air-dried silks provide a protective barrier that physically immobilized blood components with access to minimal residual moisture, in turn conferring conformational stability (34). We directly demonstrated that the physical entrapment provided by the silk matrix was effective in mitigating thermally induced degradation and we can infer there are additional benefits such as preventing exposure to enzymatic and UV stresses (26). Long-term temperature stability of dried silk formats such as films or powders should be useful for field conditions where the silk material is used as the entrapping matrix and recovery of analytes is dependent on sustained solubility, or where a dry transportable format is required before mixing with blood. In contrast, liquid plasma demonstrated instabilities after a single freeze–thaw cycle, consistent with well-established preanalytical sample management protocol (35). Previous reports have indicated that small precipitates can form in plasma isolated from heparinized blood at freezing temperatures above −80 °C (for instance the −20 °C conditions used herein), and that this is a major contributor to the loss of viability/recoverability of clotting factors in general (11). Importantly, freeze–thaw damage can be circumvented entirely using the ambient drying approach described here.

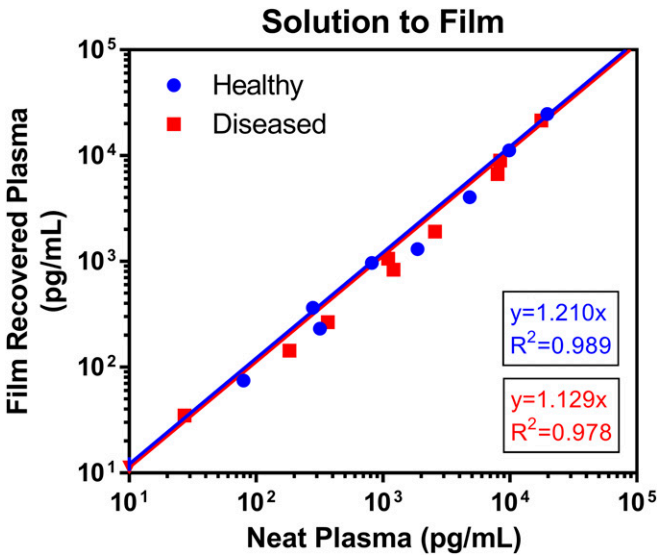

Fig. 6. Recovery of biomarkers is not dependent on patient health status. The *x* axis represents plasma levels as measured after storage at −80 °C, whereas the *y* axis represents plasma levels as measured after encapsulation in air-dried silk films. Blue data points represent readings from a healthy patient although red data points represent readings from a patient diagnosed with pancreatic cancer. Blue and red lines represent best fit lines (equations *Inset*) from linear regression. See Table S5 for raw data.

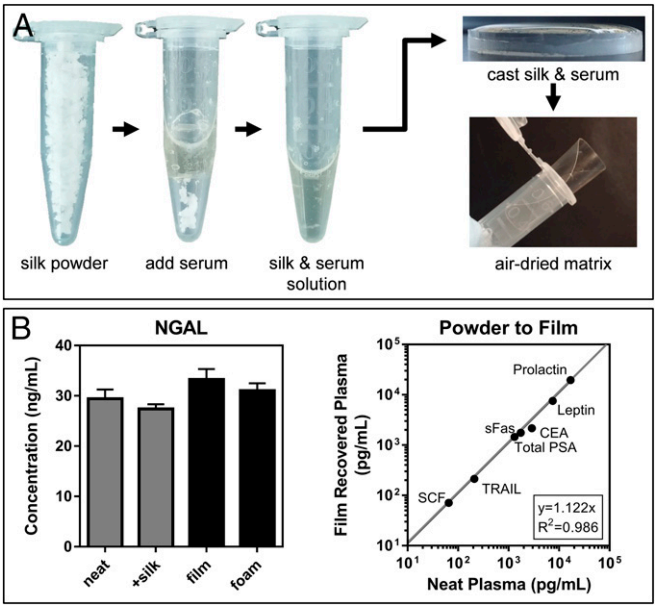

Fig. 7. Silk powder for use as a stabilizing agent in the field. (*A*) Schematic showing the regeneration of silk powder with serum to incorporate stabilizing agent. The formulation is cast and the resulting air-dried matrix placed in an Eppendorf tube. (*B*, *Left*) Plasma NGAL can be recovered after addition of 16 mg of silk powder to 50 μL of plasma diluted 8× in water ("+silk"), after air-drying the powder/plasma solution ("film"), and also after lyophilizing said the powder/plasma solution ("foam"). Data are average ± SD of $n = 4$ replicate samples from a single donor. (*B*, *Right*) Luminex data demonstrating recovery of seven biomarkers from a silk powder-generated film. Black dots indicate average ± SD of $n = 4$ replicate samples from a single donor. Gray line indicates the best-fit line (equation *Inset*) from linear regression.

In this work we used both traditional sandwich ELISA formats as well as bead-based microfluidic immunoassays for detection of the blood-based analytes, demonstrating the adaptability of the silk stabilization approach to a variety of downstream analytical techniques. In principle, the specificity of the immunoassay approach for the blood-based analytes and lack of interaction with silk protein is facilitated by the repetitive and predominately glycine–alanine–serine silk fibroin sequence (26). The same silk stabilization, recovery, and analysis techniques demonstrated here with blood should be applicable to other biospecimens, including serum, saliva, and urine (Fig. S1) as well as most other techniques used in the analytical laboratory setting (chemical assays, tandem mass spectrometry, etc.).

During the course of this study we evaluated several formulations or reconstitution alterations to improve recovery of analytes in silk-laden materials. In the context of clinical use, it is likely that several reconstitution buffers will be required per assay panel as dictated by the strength of interactions between analytes and the silk on a case-by-case basis. We anticipate that supplying a few different analyte-specific reconstitution buffers will be well-accepted in industry, assuming there is at least some broader cross-analyte compatibility. Another potential limitation inherent to drying biospecimens (whether by using a matrix or paper) is that accurate starting volumes of the specimen must be known to calculate the final sample dilution upon recovery. This can be addressed by more precisely metering the solution (capillary tube, pipetting, etc.) before mixing with the silk, as opposed to simply dabbing a heel or finger prick directly onto the substrate as is currently done on paper. The major focus of the present work was to demonstrate the multifunctional aspects of silk as a sample stabilization and transport system—both mechanical and biological in nature—although traditional pharmaceutical optimization strategies can be used in future design iterations to enhance the long-term stabilization capacity. Various

common excipients and drying techniques are compatible with silk due to the aqueous processing at ambient conditions. The solubility of silk in the presence of other entrapped materials will depend in part on the ratio or stoichiometry of the entrapped biologic to silk, the type of entrapped material, and buffers/excipients/inhibitors typically used in aiding in stabilization of these biologics. Due to the conformal nature of the protein (36) we also anticipate the compatibility of silk with a variety of commercially available collection devices.

Materials and Methods

Silk solution was prepared from the cocoons of the silkworm *Bombyx mori* as previously described (27). Films were generated by pipetting silk solution with or without deidentified blood onto PDMS surfaces and allowing solutions to dry overnight at ambient conditions. Resulting films were solubilized using water or aqueous based solutions (buffers, salts, surfactant) at designated time points, vortexed, and centrifuged. The supernatant was immediately analyzed in accordance with manufacturer assay protocols. Dried blood spots were prepared and processed following Clinical & Laboratory Standards Institute guidelines (37). Additional information on sample collection, fabrication, analytical methods, and statistical analysis is provided in *Supporting Information*.

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