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DNA preservation in silk

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Abstract

The structure of DNA is susceptible to alterations at high temperature, pH, irradiation and exposure to DNase. Options to protect and preserve DNA during storage are important for applications in genetic diagnosis, identity authentication, drug development and bioresearch. In the present study, the stability of total DNA purified from human dermal fibroblast cells as well as plasmid DNA was studied in silk protein materials. The DNA/silk mixtures were stabilized on filter paper (silk/DNA+filter) or filter paper pre-coated with silk and treated with methanol (silk/DNA+PT-filter) as a route to practical utility. After air-drying and water extraction, 50–70% of the DNA and silk could be retrieved and showed a single band on electrophoretic gels. The 6% silk/DNA+PT-filter samples provided improved stability in comparison to 3% silk/DNA+filter and DNA+filter for DNA preservation, with ~40% of the band intensity remaining at 37 °C after 40 days and ~10% after exposure to the UV light for 10 hours. Quantitative analysis using the PicoGreen assay confirmed the results. The use of Tris/Borate/EDTA (TBE) buffer enhanced the preservation and/or extraction of the DNA. The DNA extracted after storage maintained integrity and function based on serving as a functional template for PCR amplification of the gene for zinc finger protein 750 (ZNF750) and for transgene expression of red fluorescence protein (dsRed) in HEK293 cells. The high molecular weight and high content of crystalline beta-sheet structure formed on the coated surfaces likely accounted for the preservation effects observed for the silk/DNA+PT-filter samples. Although similar preservation effects were also obtained for lyophilized silk/DNA samples, the rapid and simple processing available with the silk-DNA-filter membrane system makes it appealing for future applications.

Graphical Abstract

[#]The first three authors contributed equally to this paper

silk; DNA; preservation; temperature; UV light

Silk fibroin (hereafter termed silk) is a protein biopolymer that has been widely used in biomaterials for tissue engineering and drug delivery.¹³ Silk has a block copolymer structure dominated by Gly–Ala–Gly–Ala–Gly–Ser repeats. After all-aqueous processing, silk can be readily fabricated into different material formats, such as hydrogels, tubes, sponges, fibers, microspheres, lyophilized powders and films with remarkable mechanical and biological

properties.^{14–17} Silk biomaterials are inherently stable to changes in temperature (sub zero to 200 °C)¹⁸, pH¹⁹ and moisture²⁰, and are mechanically robust due to the extensive network of physical cross-links (beta-sheets) formed during the assembly process.^{21,22} Importantly, stabilized and mechanically robust silk materials are generated without the need for any chemical or optical crosslinking and solutions and materials generated from silk can be autoclaved for sterilization if needed, a reflection of the robust material properties. Due to these features, silk biomaterials have been used as drug carriers, in medical devices, as enzyme-immobilizing materials and in many related applications.^{23–25} For example, silk was evaluated for the immobilization of glucose oxidase and had good stability during 10 months of storage at 37 °C and retained 80% of the original activity.^{26,27} These and other studies indicated that silk biomaterials are a useful matrix for immobilizing and stabilizing protein molecules due to interactions between the silk and the entrained protein molecules.

The objective of the present study was to evaluate the potential for silk materials to stabilize embedded DNA under various storage conditions of temperature and UV irradiation, with the ultimate goal to develop new silk-based DNA preservation techniques. Silk-based preservation offers advantages as a green source of the material (sericulture), relatively simple and all-aqueous and ambient material processing, and sustained preservation in mechanically robust, self-standing, or coated silk materials. In the present study, silk was coated on the surface of porous cellulose filter paper in order to facilitate post storage dissolution of silk to release the DNA embedded in the silk matrix.

MATERIALS AND METHODS

Materials

Partially degummed silk fibers were purchased from Xiehe Silk Incorporation, Shengzhou, Zhejiang province, China. Lithium bromide (LiBr) (Cat. L108934) was purchased from Aladdin (Shanghai, China). Human dermal fibroblasts (Hs 865.Sk cells) were obtained from American type culture collection (ATCC, Maryland, USA). Cell medium ingredients were purchased from Thermo Fisher Scientific (Grand Island, NY). Human genomic DNA (Cat. CW05655) and Tissue DNA kits were purchased from CWBIO (Beijing, China). DNA ladders and other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). PCR primers were purchased and DNA sequencing was performed at Genewiz (Suzhou, China). Quant-iT PicoGreen DsDNA Reagent (Cat. P11495) were purchased from Thermo Fisher Scientific (Grand Island, NY). pDsRed2-ER vectors were purchased from ClonTech (Cat. 632409, US). PolyJet™ *in vitro* DNA transfection reagent was purchased from SignaGen Laboratories (MD, US).

Purification of silk

Silk protein was purified using standard protocols as previously reported.¹³ To remove sericin contaminants from silk fibers (degumming), the cocoons of *B. mori* were cut into pieces and boiled for 30 min in 0.02 M sodium carbonate. The fibers obtained were rinsed with ultrapure water three times thoroughly and dried in a fume hood. The dried degummed silk fibers were dissolved in 9.3 M lithium bromide solution at 60°C for 4 hours, and the solution was dialyzed against ultrapure water for 3 days (water changed 3 times a day) to

remove the lithium bromide. The solution was then centrifuged to remove insoluble silk aggregates. The silk solution after dialysis (approx. 7.5 wt%) was stored at 4°C and diluted with ultrapure water before use.

Extraction of total DNA

Total DNA was extracted from the human fibroblast cell line (Hs 865.Sk cells) following the manufacturers' protocols (E.Z.N.A.TM Tissue DNA Kit, OMEGA, China). Briefly, cells (5×10^6) were resuspended with 200 μ L cold (4°C) PBS, pH 7.4, into which 25 μ L lysate solution was added and the solution was mixed by vortexing. The solution was incubated at 65°C in a water bath for 5 minutes to complete cell lysis. The solution obtained was mixed with 220 μ L DNA extraction liquid, vortexed, and incubated at 70°C for 10 minutes. After cooling to room temperature, 220 μ L of absolute ethanol was added and mixed thoroughly by vortexing. The solution was loaded onto a HiBind DNA isolation column (assembled in a 2 mL collection tube). The column was washed with the buffers provided and the DNA was eluted from the column with 100 μ L preheated (70°C) Elution Buffer. The DNA extract was stored at -20°C.

The absorbance of the DNA extracts was measured at 260 nm (A_{260}) and 280 nm (A_{280}) with a spectrophotometer (Synergy H1, BioTek, USA). The DNA concentration was calculated using the following equation: $c(\text{ng}/\mu\text{L}) = A_{260} \times 50 \times \text{dilution factor}$. The A_{260}/A_{280} ratio was used to estimate the purity of the extracted DNA, with 1.7–1.9 corresponding to 85% – 95% purity. The quality of DNA was evaluated by electrophoresis and PCR on a target gene (ZNF750 gene), and confirmed by gene sequencing.

Preparation of DNA embedded silk samples

The DNA molecules were embedded into the silk matrix in two ways – direct deposition and post-treatment deposition.

Direct deposition—The filter papers (Xin Star, Hangzhou, China) were cut into round-shape pieces with a diameter of 14 mm, which were placed into 24-well cell culture plates (Corning costar, USA). The silk solution was diluted with ultrapure water to obtain silk concentrations of 1, 3 and 6%. The extracted DNA solution was diluted with ultrapure water to 96 ng/ μ L, and 40 μ L of the DNA solution was mixed with 40 μ L silk solution. The mixed solution was pipetted on the filter paper placed in 24-well plates. The 24-well plates were placed in a fume hood overnight until the solution dried completely. The samples prepared using direct deposition are referred to as silk/DNA+filter. DNA without silk was used as control.

Post-treatment deposition—The filter papers (Xin Star, Hangzhou, China) were cut into round-shape pieces with a diameter of 14 mm, which were placed into 24-well cell culture plates (Corning costar, USA). A 100 μ L aliquot of silk solution at 1, 3, 6% was pipetted on the filter paper placed in the 24-well plates. The samples were dried in the fume hood overnight. After drying, 1 mL methanol was added into each well and the samples were incubated for 2 hours before being dried in the fume hood. The DNA solution (96 ng/ μ L, 40 μ L) was mixed with 40 μ L silk solution, and the mixed solution was pipetted on the dried

filter paper. The 24-well plates were placed in the fume hood overnight until the solution dried completely. The samples prepared using post-treatment deposition method are referred to as silk/DNA+PT-filter. DNA without silk was used as a control.

Optimization of DNA preservation by TBE during DNA entrapment

A 1×TBE (pH = 8.3), silk (3% or 6%), and DNA solution (96 ng/μL) were mixed at a volume ratio 1/1/1, with 40 μL of each solution. The 3% silk/DNA/TBE mixture was pipetted on the filter paper directly (3% silk/DNA+filter), while the 6% silk/DNA/TBE mixture was pipetted on the 6% silk coated and methanol-treated filter papers that were prepared as described above (6% silk/DNA+PT-filter). The 24-well plates were placed in the fume hood overnight until the samples dried completely. For pDsRed2-ER plasmid and its expression in HEK293 cells, 1×TBE (pH = 8.3), silk (6%), and pDsRed2-ER (250 ng/μL) were mixed at a volume ratio 1/1/1, with 40 μL of each solution. The 6% silk/DNA/TBE mixture was pipetted on the 6% silk-coated and methanol-treated filter papers that were prepared as described above (6% silk/DNA+PT-filter). The 24-well plates were placed in the fume hood overnight until the samples dried completely. DNA without silk was used as a control.

Extraction of DNA from silk-coated filter papers

DNA entrapped silk samples were prepared as described above. The samples were stored at room temperature, 37, 45°C or exposed to UV light. At designated time points (0, 3, 10, and 40 days), the samples were characterized by gel electrophoresis, Quant-iT PicoGreen, ZNF750 gene and for transfection.

Gel electrophoresis and semi-quantitative analysis

To extract DNA samples for analysis, 80 μL DI water was pipetted onto each sample. Electrophoresis was run on 0.8% agarose gels using 1× TBE buffer at 100V for 2 hours. The gels were stained with 10% (w/v) GEL RED (BIOTIUM) for 30 minutes before electrophoresis. The DNA (96 ng/μL) stored at -80°C was used as a control and the gels were imaged under UV light (245nm). The images were analyzed using Image J using a gray scale for the DNA band with a defined area calculated and converted to a peak chart. The ratio between the area of experimental sample and the area of standard sample was defined as relative gray value, while the ratio of the gray value after storage relative to that before storage was defined as DNA recovery.

Quant-iT PicoGreen DsDNA quantitative analysis

To extract DNA samples for analysis, 500 μL 1× Tris-EDTA (TE) was pipetted onto each sample. Thereafter, 100 uL of cell culture medium was transferred to a black flat bottom 96-well plate. The DNA content was determined via Quant-iT PicoGreen assay using a fluorescence spectrophotometer (Bio-TEK instrument, USA; excitation wavelength: 480 nm, emission wavelength: 520 nm).

PCR analysis and sequencing of ZNF750 gene

To extract DNA samples for analysis, 80 μL DI water was pipetted onto each sample.

PCR analysis—A short Range PCR (531 bp product from ZNF750 gene, forward primer 5'-AATACTGTGCCTCCCAGGGTAT-3', reverse primer 5'-GTACTTACCAGAGGTGGGCAGTG-3') was performed to verify the integrity of DNA samples. Each reaction sample consisted of 5 µL of 10×Superstar Taq PCR Buffer (containing 100 mM KCl, 40 mM Tris-HCl), 10 mM deoxynucleoside triphosphates [dNTPs], 1 U Superstar Taq DNA polymerase, 400 nM forward primer, 400 nM reverse primer, 5 µL of template DNA (45 ng/µL), and RNase-Free H₂O. The total reaction volume used for the PCR was 50 µL. The PCR program used was: initial denaturation 9 °C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 60 s. The quality of PCR products was assessed on a 1.5% agarose gel, stained with 10% GEL-RED (BIOTIUM, US).

Gel extraction and gene sequencing—Gel extractions were performed manually according to the manufacturer's protocols (DNA purification Kit, CWBio, China). Briefly, the gel slice containing the PCR product was excised with a scalpel and placed in a tube. Based on the weight of the gel slice, approximately 3 volumes of gel melting buffer was added to 1 volume of gel. The sample was incubated at 50°C for 10 minutes to dissolve the gel. After the gel slice was dissolved completely, the solution was loaded onto a spin column provided in the kit. The column was washed with buffer provided to remove impurities, and the purified DNA in 50 µL of elution buffer was obtained at the end and was subjected to DNA sequencing (Genewiz, Suzhou, China). DNA sequences were analyzed using National Center for Biotechnology Information (NCBI) database to evaluate the sequence consensus with the target gene (ZNF 750).

Transgene expression of pDsRed2-ER plasmid DNAs

HEK293 (American Type Culture Collection, ATCC) cells were amplified in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified air incubator containing 5% CO₂. When the cell density reached approximately 80% confluence, the cells were harvested, trypsinized, washed and seeded onto 6-well plates (Corning, USA) with 5 mL of cell culture medium in each well. Cell seeding density was approximately 1×10⁵ cells per well. 1× TE (100 µL) was pipetted onto each sample to extract the DNA. When the cell density reached approximately 60% confluence, plasmids were transfected into HEK293s following manufacturer's protocol. Briefly, solution A (10 µL DNA extraction solution and 100 µL free FBS/DMEM) was added to solution B (4 µL PolyJet™ *in vitro* DNA transfection reagent and 100 µL free FBS/DMEM). The mixture was vortexed, incubated at room temperature for 15 min, mixed with medium (1 mL) and subsequently applied to the cells. Cell culture medium was changed after 6 h incubation and 28 h after transfection. Live GFP pictures were acquired with a Leica fluorescence microscope. pDsRed2-ER was excited at a wavelength of 568 nm, and emitted light was collected between 570 and 620 nm.

Statistical analysis

Results are expressed as means ± standard deviations. Statistical differences between samples were evaluated by SPSS (16.0) using a one-way student's t-test. Differences were considered significant when $p < 0.05$, and very significant when $p < 0.005$.

RESULTS

Optimization of DNA extraction from filter papers

Pre-mixing silk and DNA samples improved DNA extraction efficiency—The DNA samples were mixed with silk at different concentrations and the mixture was deposited on the filter papers for 24 hours at room temperature. The quality (degradation bands) as well as semi-quantitation (band intensities) of the DNA samples were assessed by electrophoresis after extraction of the DNA. Clear DNA bands without degradation were seen for the recovered filter paper samples containing 1, 3, 6% silk, while bands were not seen for samples without silk (Figure 1(A)). The control solution samples (no filter) showed bands with similar brightness in Figure 1(A) (lower panel) and similar relative gray values in Figure 1(B). The relative gray values of 3% silk/DNA+filter and 6% silk/DNA+filter were appr. 0.9 and 0.7, respectively, significantly higher than that of the DNA alone sample (appr. 0.4, $p < 0.005$, Figure 1(C)). The DNA recovery of 3% and 6% silk samples were 82% and 77%, respectively, also significantly higher than that of the DNA alone sample ($p < 0.005$, Figure 1(D)). These results indicated that silk protected the DNA from degradation during sample preparation and extraction. The best concentration of silk for protection of DNA for the ranges studied here was 3%.

Pre-treatment of filter papers with silk and methanol improved DNA extraction efficiency—Methanol treatment induces a structural transition of silk materials from random coil-dominated to beta sheet-dominated structures, resulting in decreased solubility of the silk and increased enzymatic degradation time and mechanical stiffness.²⁸ This was confirmed in the present study when 3% and 6% silk solution was air-dried into films that were subsequently treated with methanol and subjected to FTIR measurement. As shown in Figure S1, the methanol-treated films showed greater than 50% beta-sheet structure content while the control samples (untreated films and lyophilized solution) showed 20–25% beta-sheet content. We expected high beta-sheet content of silk may provide a stabilizing matrix for DNA molecules, as indicated in our previous enzyme stabilization studies,²⁷ while the random coil structure of silk may facilitate DNA extraction, as confirmed in Figure 1. In the present study, when DNA was deposited on the pretreated silk filters (1, 3 and 6% silk-coated filter papers soaked in 1 mL methanol for 2 hours to induce beta-sheet formation), the 6% silk-coated and methanol-treated filter sample showed significantly higher relative gray values (~ 0.6) and extraction recovery (~ 50%) than the other samples (Figure S2). When 1, 3 and 6% silk/DNA mixtures were deposited on the corresponding pre-treated filters, the 3% silk/DNA+PT-filter and 6% silk/DNA+PT-filter samples had relative gray values (~ 0.8) and extraction recoveries (~ 70%), significantly higher than the DNA alone sample ($p < 0.005$, Figure 2). Methanol treatment to induce silk beta-sheet structure in the pre-coated filters did not significantly influence DNA extraction efficiency (comparing Figure 1(D) and Figure 2(D)). From the above extraction experiments, we chose two conditions to prepare samples for the following studies: (1) 3% silk/DNA+filter; (2) 6% silk/DNA+PT-filter.

Using TBE buffer improved DNA stabilization—TBE is used as a buffer in electrophoresis and has electrical conductivity advantageous to the migration of the DNA. TBE consists of EDTA, Tris and boric acid and the EDTA can chelate magnesium ions. The

buffer also can prevent the activation of DNA enzymes during electrophoresis. Tris is commonly used as a biological buffer.^{29–31} The effect of adding TBE in the silk/DNA mixture on DNA preservation was evaluated. The samples were stored at different temperature for 24 hours before being analyzed. As shown in figure 3, the use of TBE in sample processing significantly enhanced DNA stability compared with the samples without TBE for all three temperatures tested (Figure 3). The effect of TBE became more pronounced with increased storage temperature. The relative gray value of 3% silk/DNA/TBE+filter samples stored at 45°C was ~0.7, while that of the 3% silk/DNA+filter was only 0.1 (Figure 3(D)). For all the following long-term preservation studies, TBE was used for sample preparation.

DNA preservation under different temperatures

DNA integrity by gel electrophoresis—Filter papers containing DNA samples were stored at room temperature, 37°C and 45°C for 3, 10 and 40 days, and DNA integrity was qualitatively analyzed by electrophoresis at each time point. The control DNA (no silk) degraded rapidly on filter paper, with a relative gray value of ~0.2 at day 3 when stored at room temperature and 45°C, and ~0.35 when stored at 37°C (Figure 4). In contrast, the 3% silk/DNA+filter sample had the relative gray value of 0.4–0.5 under all three temperatures at day 3, significantly higher than the control as well as the 6% silk/DNA+PT-filter ($p < 0.005$, Figure 4). At day 10, the relatively gray values determined for all the samples stored at room temperature were higher than those determined at day 3 (Figure 4B). The relative gray values for 3% silk/DNA+filter and 6% silk/DNA+PT-filter were ~0.6, significantly higher than that of the control ($p < 0.005$). For 37°C and 45°C, the relative gray values of the two silk samples were also significantly higher than the control ($p < 0.005$), but the difference was not as pronounced as that at room temperature (Figure 4). At day 40, no significant differences were seen for the samples stored at room temperature and 45°C. Interestingly, the 6% silk/DNA+PT-filter sample stored at 37°C still showed a clear DNA band (Figure 4A) and the relative gray value was ~0.4, significantly higher than that of the 3% silk/DNA+filter (0.3) and the control (0.1) ($p < 0.005$).

DNA stability assessed by PicroGreen DsDNA quantitation: The stability of silk-embedded human genomic and plasmid DNA was assessed by Quant-iT PicroGreen DsDNA assay. The DNA-containing silk films prepared on filter papers were incubated at room temperature, 37 and 45 °C before the DNA was extracted and subjected to analysis. Figure 5 shows the percentage of DNA remained with respect to the equivalent amount of DNA freshly prepared. The results confirmed the conclusion obtained from gel electrophoresis (Figure 4) that with the post-treatment deposition (6% silk/DNA+PT-filter), more than 80% genomic DNA can be preserved at different temperatures for at least 40 days, whereas the control samples without silk protection lost almost 80% DNA content. Plasmid DNA followed a similar trend of preservation based on the data shown in Figure 5B.

Amplification of the target gene using silk-preserved DNA as template—The zinc finger protein 750 (ZNF750) controls epithelial homeostasis in the body. Mutations in the gene for this protein are associated with cancer and psoriasis.³² In the present study, the gene for ZNF750 was used as a target to demonstrate the function of DNA after storage and

reconstitution. PCR of the ZNF 750 gene (531 bp) was performed on the DNA stored at different temperatures. When the solution samples (DNA alone) were used as templates for PCR, a clear bright band at ~500 bp was seen on the gel electrophoresis (Figure 6, room temperature 0 h, solution), indicating that the DNA was functional. When 3% silk/DNA +filter was dried out at room temperature and the DNA was extracted for PCR, a similar band was seen (Figure 6, lane 4), indicating that drying samples on the filter paper and the subsequent extraction had no impact on the PCR results. PCR was further performed on the filter paper samples as well as the controls of lyophilized powder after high temperature treatment (45 °C, 24 hours). As shown in Figure 5, the 3% silk/DNA+filter sample as well as the lyophilized DNA and 3% silk/DNA powder all showed a band around 500 bp with similar intensities, which was stronger than that of the control (DNA+filter). Thus, DNA preserved in the presence of silk was biologically functional as a PCR template.

DNA sequences obtained from the PCR products were compared with that from the National Center for Biotechnology Information (NCBI) database to obtain gene identities and gaps (Table 1). For the solution samples, the DNA sample and 3% silk/DNA samples shared more than 99% identities, indicating that the PCR products shown in Figure 5 were indeed the gene sequence for ZNF750. The presence of silk did not impact either the PCR process or the subsequent gene sequencing. The gaps (missing bases in the sequencing data, not shown) of 3% silk/DNA samples, either in solution or loaded on the filter paper, were zero. After incubation at 45°C for 24 hours, the 3% silk/DNA+filter sample showed 100% identity with the sequence of ZNF750 gene. The three types of lyophilized powder (DNA alone, 3% silk/DNA, 6% silk/DNA) all had 99% identities, with gaps of 1, 2, 1, respectively. Compared to the silk-preserved DNA, lyophilization can also protect DNA from high temperature damage, although the quality was slightly worse than the silk-preserved samples (Table 1).

DNA preservation under UV irradiation

Gene integrity examined by gel electrophoresis—Three kinds of filter paper samples (DNA, 3% silk/DNA+filter, 6% silk/DNA+PT-filter) were prepared and exposed to UV light (245 nm) for 1, 2, 10 hours. As shown in Figure 6(A), the control of DNA+filter thoroughly degraded after 1 hr exposure, while the 3% silk/DNA+filter and 6% silk/DNA +PT-filter both showed bright bands even after 2 hours UV irradiation. The bright band of 6% silk/DNA+PT-filter sample still remained even after 10 hours UV irradiation. The relative gray values determined showed the same trend as the band intensities, with that of the 6% silk/DNA+PT-filter (~0.1) significantly higher than the 3% silk/DNA+filter and the control (DNA+filter) at all the time points (Figure 7(B)). Thus, DNA was protected from UV light in the 6% silk/DNA+PT-filter sample, consistent with the result in the temperature experiment.

Amplification of the target gene using silk-preserved DNA as a template—The ZNF750 gene in the DNA samples exposed under UV irradiation for 2 hours were examined by PCR. The control sample (DNA without UV exposure), 3% silk/DNA+filter, 6% silk/DNA+PT-filter, lyophilized DNA, and lyophilized 6% silk/DNA samples after UV exposure for 2 hours all showed a clear band around 500 bp, indicating the DNA was protected from UV irradiation in these samples to allow successful amplification of the

ZNF750 gene (Figure 7). In contrast, the DNA+filter and 3% silk/DNA+filter samples after UV exposure for 2 hours did not show a clear band on the gel (Figure 7). DNA was therefore damaged in these samples, consistent with the result from gel electrophoresis.

DNA sequences obtained from the PCR products above were compared with that from the NCBI database to obtain gene identities and gaps. For all the samples that could be cut out of the gel in Figure 8 (control DNA, 6% silk/DNA+PT-filter, lyophilized DNA, lyophilized 3% silk/DNA and lyophilized 6% silk/DNA), the gene identities were 99% and the gaps were 1 or 2, similar to the control samples of DNA and silk/DNA in solution (Table 2).

To demonstrate the biological function after storage, the extracted plasmid DNA (pDsRed2-ER plasmids) was tested for transgene expression in HEK293 cells with control of newly prepared plasmid DNA. As shown in Figure 9, plasmid pDsRed2-ER coding red-fluorescent protein (dsRed) fused with the KDEL sequence can target endoplasmic reticulum (ER), showing bright red. Red-fluorescent protein was investigated by fluorescence microscopy (Figure 9). Fluorescence microscopic images showed that, even after long-term storage at different temperatures, dsRed expression did not decline when compared with the newly prepared plasmid, while the samples without silk protection had very low dsRed expression.

DISCUSSION

The goal of the present research was to develop a new technology by which DNA samples collected from research labs or hospitals can be rapidly treated on site with no need of using special instruments or detrimental reagents. The treated samples can be stored and transported at ambient conditions, and can be readily analyzed upon reconstitution in water. Such a strategy has been exploited previously. Some commercial products, such as FTATM ClassicCard (Whatman), SampleMatrix (Biomatrix), and GenTegra (GenVault), can preserve purified DNA at room temperature for long periods of time.³³ FTATM ClassicCard is coated with chemicals that lyse cells, denature proteins and entrap DNA in the matrix fiber, allowing for easy transport and long-term storage of DNA.^{34,35} However, the extraction method to recover the DNA from the product is complex: DNA-containing samples need to be soaked in a special FTA purification solution provided by the manufacturer and further extracted by TE buffer. Matrix relies on a glass polymer that protects DNA from heat and UV light through a mechanism similar to that used by extremophiles, small organisms that can survive in dry environments for up to 120 years.³⁶ DNA samples need to be dried after being added in this polymer material. The major ingredient of GenTegra is an inorganic mineral matrix with oxidation protection and antimicrobial activity. For both GenTegra and SampleMatrix, the DNA needs to be stored in a special tube, and the major functional ingredients, the products are expensive and each kit can only handle a limited amount of DNA (30 µg). To overcome these drawbacks and developed a more robust and more user friendly DNA preservation technology, we chose to use silk.

Silk-stabilized labile molecules, such as enzymes and nutraceuticals, can preserve their bioactivities for several months at high temperatures.^{37–40} Silk materials used in these studies were mainly air-dried films. Drying process usually takes a few hours to overnight depending on the film thickness and time/temperature used. Longer drying time is

disadvantages for preserving the bioactivities of the incorporated molecules. Depending on the film thickness obtained and the time used for drying, the films obtained can be fully soluble or partially soluble in water. The undesired silk beta-sheet structure formation and strong intermolecular interaction between silk molecules account for the film insolubility after drying. In the present study, we attempted to use highly porous filter paper which was coated with silk. Due to the high surface area obtained, DNA solution or silk/DNA mixed solution could be quickly dried (within a few minutes) in the filter cellulose matrix with a minimal amount of silk beta-sheet structure formation and could be easily re-dissolved in water to obtain silk solution containing bioactive molecules. To achieve the highest extraction efficiencies for both silk and DNA, we treated the samples as follows: (1) Pre-mixing silk with DNA prior to drying on the filter paper matrix. Silk molecules may help reduce shear-induced degradation and maintain integrity of DNA molecules during air-drying. (2) Pre-coating filter paper with beta-sheet-structure-rich silk. Highly stable beta-sheet structure of silk may help immobilize DNA molecules in the matrix and shield them from DNase attacking. (3) Using TBE buffer instead of water to help stabilize DNA molecules during drying process. Various silk concentrations were tested during these optimizations. Two sample preparation conditions (3% silk/DNA+filter and 6% silk/DNA+PT-filter) were chosen at the end for the preservation studies. Under these conditions, 50–70% of the original loaded DNA and silk could be retrieved and showed a single band on electrophoretic gels.

In the long term preservation study under different temperatures, the 6% silk/DNA+PT-filter samples provided improved stability in comparison to 3% silk/DNA+filter for DNA preservation, with ~40% of the band intensity remaining at 37 °C after 40 days. Interestingly, compared with the other two storage temperatures (room temperature and 45°C), samples stored at 37 °C showed the most consistent preservation (Figure 4). The same samples stored at room temperature showed the best preservation effects within 10 days, but the DNA levels dropped largely after 40 days storage, lower than those stored at 37°C (Figure 4B). While the mechanism of long term DNA preservation at 37 °C for the 6% silk/DNA+PT-filter sample is not clear, 37 °C may promote stronger interactions between the DNA and silk, especially at the beta-sheet regions. Based on our prior studies with protein stabilization in silk, where mechanistically a combination of hydrophobic and charge interactions played an important role, each of these factors may play a subtle role in the case of DNA. After long term storage at different temperatures, genomic DNA was subjected to PCR analysis for the gene of zinc finger protein 750 (ZNF750), and plasmid DNA was subjected to cell transfection for the red fluorescence protein (dsRed) expression. The results demonstrated that the DNA preserved in the presence of silk, even at high temperatures, still functioned as PCR templates and maintained biological activity in terms of transgene expression. In these studies, silk/DNA mixed solution was also lyophilized instead of being dried on the filter papers, and the DNA preservation in these lyophilized samples was evaluated. Clearly, lyophilization could also preserve DNA to the same level as the silk-coated polymer matrix (Figure 6). Since lyophilization needs special equipment (lyophilizer) and a relatively long time for sample preparation, the method of rapid drying of DNA in the presence of silk on filter paper should be preferred in many future applications.

When the samples were exposed to the UV light for 10 hours, the 6% silk/DNA+PT-filter samples also showed improved DNA preservation when compared to 3% silk/DNA+filter and DNA+filter. It has been known that silk can absorb UV light due to its amino acid composition rich of tyrosine, thus being widely used in cosmetic products. Our data demonstrated that silk could protect DNA from UV damage for extended time frames (>10 hours), consistent with previous reports. These findings are also consistent with our prior observations on tetracycline antibiotic protection in silk upon exposure to light.³⁸

Mechanistically, compounds are most often stabilized in silk via a combination of hydrophobic interactions and charge complexation.^{39,40} In the case of DNA, the negative charge would suggest limited charge-charge complexation in the present study as silk has a net negative charge at the pH studied here. Thus the assumption is that some degree of hydrophobic interaction may play a role, or some degree of pi-pi stacking with the predominant tyrosines present in the silk (~5% from gene sequence prediction and amino acid residue analysis)^{41, 42}. As reported in the literature, aromatic amino acids including tyrosine could protect bacteriophage Y2 from inactivation by UV-light⁴³. The abundant and inexpensive silk material obtained via the textile industry via sericulture, the simple and all-aqueous process used here, and the diversified material formats available with silk materials provide an alternative for DNA preservation in a variety of applications.

CONCLUSIONS

The present study demonstrated that silk-coated filter papers could be used to preserve DNA at high temperatures (37, 45 °C) for more than 40 days and under UV irradiation for more than 10 hours. Approximately 70% of the silk could be extracted from filter paper by pipetting and the DNA extracted (~ 50% of total amount) preserved its integrity as demonstrated by PCR, sequence analyses and pDsRed2-ER plasmid transfection. Use of TBE buffer during sample drying enhanced the preservative. Among various samples being tested, higher silk concentration (6%) and methanol treatment showed the best preservation effect, likely because of the high content of beta-sheet structure which promoted interactions between silk and the DNA. Silk materials provide a new option for DNA preservation when compared to currently available methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1. Impact of different silk concentrations on DNA extraction

A, lanes 1, 2: control DNA samples (96 ng/ μ L) stored at -80°C ; lanes 3, 4: DNA+filter; lanes 5, 6: 1% silk/DNA+filter; lanes 7, 8: 3% silk/DNA+filter; lanes 9, 10: 6% silk/DNA+filter; lanes 3, 5, 7, 9: DNA extracted from the filter papers; lanes 4, 6, 8, 10: same amount of silk/DNA solution loaded directly to the gel. B, relative gray value of silk/DNA mixtures (lower panel in A). C, relative gray value of DNA extracted from silk filter paper (upper panel in A). D, extraction recovery of DNA from silk filter paper. N = 4. **indicates very significant difference ($p < 0.005$) between groups. SF = silk fibroin.

FIGURE 2. DNA extraction from the pre-treated filter paper after deposition of DNA/silk mixed solution

A, gel electrophoresis on DNA samples. Lanes 1, 2: control sample (DNA solution (96 ng/μL) stored at -80°C); lanes 3, 4: DNA+filter; lanes 5, 6: 1% silk/DNA+PT-filter; lanes 7, 8: 3% silk/DNA+PT-filter; lanes 9, 10: 6% silk/DNA+PT-filter; lanes 3, 5, 7, 9: DNA extracted from the filter papers; lanes 4, 6, 8, 10: same amount of silk/DNA solution loaded directly to the gel. B, relative gray values of the DNA/silk solution samples (lower panel in A). C, relative gray value of DNA extracted from the filter papers (upper panel in A). D, extraction recovery of DNA from the filter papers. N = 4. **indicates significant difference ($p < 0.005$) between groups. SF = silk fibroin.

Figure 3.

Preservation of DNA in the presence of TBE. A, lanes 1, 5: control samples (DNA solution (96 ng/ μ L) stored at -80°C); lanes 2, 6: DNA+filter; lanes 3, 7: 3% silk/DNA+filter; lanes 4, 8: 6% silk/DNA+PT-filter. All filter paper samples were extracted by $1\times$ TBE buffer, pH 8.3. B, relative gray value of samples at room temperature. C, relative gray value of samples at 37°C . D, relative gray value of samples at 45°C . N = 4. **indicates significant difference ($p < 0.005$) between groups.

FIGURE 4. Long-term DNA preservation at different temperatures

The filter papers loaded with silk and DNA were incubated at different temperatures for 3, 10, and 40 days. A, gel electrophoresis on DNA samples. Lanes 1, 4, 7: DNA+filter; lanes 2, 5, 8: 3% silk/DNA+filter; lanes 3, 6, 9: 6% silk/DNA+PT-filter. B, C, D: Relative gray value of samples at room temperature, 37°C and 45°C, respectively. DNA was extracted by using TBE buffer, pH = 8.3. N = 4. **indicates significant difference ($p < 0.005$) between groups.

FIGURE 5. Long-term human genomic (A) and plasmid (B) DNA preservation at different temperatures

The filter papers coated with silk (6%) and DNA were incubated at different temperatures for 3, 10, and 40 days (room temperature, 37°C and 45°C). DNA was extracted by using TE buffer, pH = 8.3. N = 4.

FIGURE 6. Gene integrity of silk/DNA stored 45°C

The DNA was preserved at 45°C in filter papers and lyophilized powder for 24h before being extracted for PCR analysis on ZNF750 gene. Lane 1: DNA ladder; lane 2: control DNA (96 ng/μL) stored at -80 °C; lane 3: DNA+filter; lane 4: 3% silk/DNA+filter; lanes 5: 6% silk/DNA+PT-filter; lane 6: lyophilized DNA; lanes 7: lyophilized 3% Silk/DNA; lanes 8: lyophilized 6% Silk/DNA.

FIGURE 7. DNA preservation under UV exposure
The DNA samples were irradiated by UV light for 1, 2, 10 h. A, gel electrophoresis on the DNA extracted from the filter paper. Control sample: DNA solution (96 ng/ μ L) stored under -80°C . B, relative gray values of the filter paper samples under UV irradiation. N = 4. **indicates significant difference ($p < 0.005$) between groups.

FIGURE 8. Gene integrity of the DNA samples exposed under UV exposure

The samples were exposed to the UV light for 2 h before being extracted for PCR analysis on ZNF750 gene. Lane 1: DNA ladder; lane 2: control DNA (96 ng/ μ L) stored at -80°C ; lane 3: DNA+filter; lane 4: 3% silk/DNA+filter; lanes 5: 6% silk/DNA+PT-filter; Lane 6: lyophilized DNA; lanes 7: lyophilized 3% silk/DNA; lanes 8: lyophilized 6% Silk/DNA.

FIGURE 9. Long-term plasmid DNA activity at different temperatures

The filter papers coated with silk (6%) and plasmid DNA (pDsRed2-ER) were incubated at different temperatures (room temperature, 37°C and 45°C) for 3 days. Plasmid DNA was extracted with PBS buffer, pH = 7.4, and was used to transfect HEK293 cells. Bar: 100 μm.

TABLE 1
Identities and gaps of gene sequencing of samples after high temperature damage. Give details of process, etc.

Sample	Solution		Filter paper			Lyophilized powder		
	DNA	3% silk+DNA	3% silk+DNA	6% silk/DNA+PT-filter	DNA	3% silk+DNA	6% silk+DNA	
Identities	99%	100%	100%	99%	99%	99%	99%	
Gaps	2/533	0/498	0/510	1/521	1/521	2/523	1/521	

TABLE 2

Identities and gaps of gene sequencing of samples after UV irradiation damage.

Sample	Solution		Filter paper		Lyophilized powder		
	DNA	3% silk +DNA	6% silk /DNA+PT-filter	99%	DNA	3% silk +DNA	6% silk +DNA
Identities	99%	100%	99%		99%	99%	99%
Gaps	2/533	0/498	2/521		0/519	2/533	1/533