Rheological profiles of DNA extracts from forensic bloodstain samples

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Abstract:

Rheology can be used to probe the differences in physical properties and in-solution behaviours of synthetic and biological polymers, like deoxyribonucleic acid (DNA). Large fragments of highly concentrated genomic and phage DNA have been characterized using rheology; however, this amount and size of DNA are atypical in DNA extracted from forensic evidence. To determine the applicability of rheology for the analysis of dilute concentrations of short DNA fragments expected from forensic specimens (low molecular weight DNA at concentrations in the ng/µL range), we conducted an optimization experiment in which we varied the size, concentration, and conformation of synthetic DNA in various solvents and polymer matrices. We found that incorporating DNA into an alginate-based, ionically crosslinked hydrogel produced the greatest differences in rheological profiles from synthetic DNA comprised of different physical properties. The conformation and size of encapsulated DNA provided significantly different responses during dynamic oscillatory measurements (p<0.05). Additionally, a time since deposition (TSD) study was performed using rotational and oscillatory tests to understand the changes in DNA extracts from bloodstains left to degrade for up to 19 months. DNA extracted from all timepoints could be detected and quantified for the tested temperature conditions (-20°C, 4°C, 22°C). Statistical analyses revealed moderate correlations between the rheological responses of DNA-containing materials and TSD ($r = -0.57$ to $r = 0.62$). Our results highlight the viability of rheology as a technique for the analysis of dilute DNA oligos and DNA extracts, and as a complementary technique for the determination of bloodstain TSD.

Keywords: biopolymer, hydrogel, dilute regime, strain overshoot, time since deposition, bloodstain
1. Introduction
Deoxyribonucleic acid (DNA) is a double-stranded biological polymer composed of repeating units (nucleotides) that typically form a double-helical structure responsible for the semi-flexible nature of DNA\(^4\). Each nucleotide is composed of a nitrogenous base (i.e., adenine, cytosine, guanine, and thymine), a deoxyribose sugar and a phosphate group. DNA is a polyelectrolyte: each nucleotide is negatively charged due to the phosphate backbone, providing DNA molecules with a net negative charge\(^5-8\). DNA is an important biomolecule as it contains the genetic information required for the functioning, development, and reproduction of an organism\(^1\). In addition to its critical biological role, DNA displays important physicochemical properties and has increasingly been used as a model polymer in polymer physics studies\(^4,9-11\). DNA is easily fluorescently labeled, stained, and visualized\(^12,13\). It displays high monodispersity in solution and can be synthesized with different conformations over a large size range\(^4,12,14,15\). Not only can DNA help model the behaviour of other polymers in solution, but elucidating its macro- and microscopic behaviour in solution is crucial for the fundamental understanding of molecular biology, biophysics and development of relevant biological applications, such as microfluidic and nanofluidic "lab-on-chip" devices\(^12,14,16,17\). Importantly, the typology, conformation, size, molecular weight and concentration of DNA will influence its behaviour in solution, leading to different rheological responses\(^5,5,24,25,14,15,18-23\). Electrostatic interactions with counterions in solution also impact the properties and behaviour of DNA; in these scenarios, DNA behaves similarly to neutral synthetic polymers\(^5,6,8,9\). Bulk rheological measurements are commonly obtained through flow (extensional and/or shear) and dynamic oscillatory testing.

Flow sweeps are performed to obtain the viscosity and flow profile of a material. High molecular weight (HMW) DNA displays shear-thinning behaviour as the rate of shear increases\(^14\). DNA solutions often demonstrate a first Newtonian plateau, where viscosity is independent of the rate of shear until a critical shear rate (\(\dot{\gamma}_c\)), at which point the polymer begins to flow, decreasing the viscosity. For same-sized DNA, a greater concentration results in a greater apparent shear rate viscosity (\(\eta_0\))\(^4,14\). Dakhil et al. analyzed double-stranded \(\lambda\)-DNA (48.5 kbp) at concentrations ranging from 0.02 – 0.315 mg/mL and revealed a positive linear relationship between the infinite shear-viscosity and DNA concentration\(^14\). Bravo-Anaya et al. found a similar positive relationship between DNA concentration and steady-state viscosity: greater concentrations of calf-thymus DNA produced larger viscosity values\(^2\). Pan et al. also demonstrated this using 25 kbp DNA extracted from \(E. coli\), \(\lambda\)-DNA and T4 bacteriophage (165.6 kbp) DNA\(^20\). The influence of concentration can likely be attributed to the overlap concentration (\(c^*\); polymer concentrations below this fall in the dilute regime, while those above are either in the semi-dilute or entangled regime\(^4\). Individual polymer chains in the dilute range do not interact with each other while those above \(c^*\) overlap and can become entangled at sufficiently high concentrations\(^2,4\). Molecular weight and size also influence the flow profile of DNA solutions; in another study by Bravo-Anaya et al., low molecular weight salmon sperm DNA displayed Newtonian behaviour during flow sweeps, while high-molecular weight calf-thymus DNA exhibited shear-thinning behaviour\(^21\). In addition, Pan et al. demonstrated that critical shear rate was positively correlated to molecular weight: larger fragments of DNA required greater rates of shear to flow\(^20\).

Dynamic oscillatory testing, such as strain amplitude sweeps and frequency sweeps, can probe the microstructure, linear viscoelasticity (LVE), and nonlinear and time-dependent behaviour of DNA in solution\(^5,18,26\). DNA concentration influences the rheological responses observed in oscillatory testing. Mason et al. characterized different concentrations of calf-thymus DNA (13 kbp) in a saline buffer using strain amplitude sweeps: a greater DNA concentration increased the storage (\(G'\)) and loss (\(G''\)) moduli\(^18\). However, critical strain values and linear viscoelastic ranges (LVRs) remained similar, irrespective of DNA concentration\(^18\). The modulus crossover in frequency sweeps occurred at lower frequencies for more concentrated DNA solutions, which was also observed by Boukany et al., Bravo-Anaya et al. and Banik et al.\(^2,4,18,22\). DNA conformation plays a key role in the rheological response during dynamic testing. Goudoulas et al. demonstrated that similarly concentrated single-stranded DNA (ssDNA; ~50 kbp, 11 mg/mL) and double-stranded DNA (dsDNA; ~115 kbp, 13 mg/mL) demonstrated different linear and non-linear
viscoelastic behaviours\textsuperscript{5}. ssDNA had larger G' values in the LVR than dsDNA and displayed strain overshoot at intermediate strain values while dsDNA did not. ssDNA also had a much larger G' at low frequencies during frequency sweeps than dsDNA, which can be attributed to the lower persistence length (and increased flexibility) of ssDNA\textsuperscript{5}.

As illustrated, DNA rheology work to date has mostly focused on large and highly-concentrated fragments of DNA – however, DNA is also found in lower concentrations as shorter fragments and as mixtures of fragments with high polydispersity, especially when extracted from ex vivo biological samples. Little work has been completed to explain the behaviour of short, dilute fragments of nucleic acids in solution, even with the broad biomedical applications provided by short oligos\textsuperscript{27,28}. Polydisperse mixtures of DNA, such as genomic DNA extracts, are rarely probed to determine their dynamics in solution despite their importance in genomics and forensics. Low amounts (ng/\mu L) of short DNA fragments and DNA extracts, dubbed forensically relevant DNA in this study, is of particular interest to forensic scientists; source attribution of biological specimens\textsuperscript{29,30}, the obtainment of phenotypic information\textsuperscript{31,32} and possible relationships with time since deposition (TSD)\textsuperscript{33–35} can be achieved through DNA evidence. DNA extracted from crime scene specimens represents a very dilute and degraded sample when compared to solutions characterized using rheology in the literature. Therefore, we might not observe a strong rheological response from such low levels of DNA (i.e., DNA in the dilute regime) on its own. Rather, we hypothesized that by incorporating dilute DNA extracts into stable polymeric systems or viscous solutions (i.e., matrices), we could observe changes to the viscoelastic and mechanical properties of those matrices. By quantifying the influence of DNA on the recorded rheological response, we could potentially infer how the physical properties and rheological behaviour of DNA change with TSD.

TSD refers to the time that has elapsed since the creation/deposition of biological evidence, such as biological fluids, at a crime scene. For example, as a bloodstain ages (i.e., TSD increases), DNA undergoes fragmentation through physicochemical means\textsuperscript{36}, producing fragments of smaller sizes. Degradation via fragmentation can occur through enzymatic activity, where endo- and exonucleases cleave the phosphodiester bonds of the DNA backbone\textsuperscript{36,37}. Most effective enzymatic activity occurs under hydrated environments, suggesting reduced activity as the bloodstain ages\textsuperscript{37}. Hydrolysis reactions are also prevalent; the N-glycosidic bond between the deoxyribose sugar and the nitrogenous bases is cleaved, resulting in rapid depurination and the formation of abasic sites\textsuperscript{38}. Deamination of cytosine is another rapid reaction, resulting in the formation of uracil and leading to further structural instability\textsuperscript{39,40}. Oxidative stress and production of reactive oxygen species (ROS) cause nucleotide damage and single-stranded nicks or double-stranded breaks\textsuperscript{41} while alkylating agents and UV irradiation produce additional ROS and promote crosslinking, chain breaks and dimerization of pyrimidines\textsuperscript{42,43}. The external stresses on the DNA molecules destabilize the naturally occurring base-pairing, leading to fragmentation and continued degradation. Therefore, a technique that can discriminate between different physical properties of DNA could prove to be very useful in TSD studies\textsuperscript{36}. Selected matrices should display different interactions with DNA due to varying physical and chemical properties. Briefly, TE-Buffer was chosen as a matrix that would minimize DNA interaction and provide a baseline for DNA behaviour as it is often used as a solvent in DNA rheology studies\textsuperscript{2–24}. A silica nanoparticle solution was chosen due to DNA’s binding affinity for silica\textsuperscript{44}; ssDNA has demonstrated stronger attraction to silica than dsDNA due to its flexibility and increased opportunity for hydrophobic interactions between unpaired nitrogenous bases and the silica surface\textsuperscript{44}. Alginate can form various hydrogels via crosslinking with divalent cations like Ca\textsuperscript{2+} and Mg\textsuperscript{2+}\textsuperscript{45}. I onically crosslinked alginate hydrogels were selected as they can encapsulate large molecules with fast and non-hazardous gelation while demonstrating greater viscoelasticity than TE buffer/silica\textsuperscript{46,47}. Methylcellulose is an amphiphilic cellulose derivative in which methyl groups substitute the hydroxyl groups at certain carbon positions\textsuperscript{48}. This substitution pattern decreases the stability of the compound and prevents chain-chain packing\textsuperscript{48}. It provides us with another gel-like matrix, although there are no ionic interactions in these gels.
2. Experimental

PART I: SINGLE DNA FRAGMENTS

Materials
We selected six matrices to determine the material which would provide the best resolution between differing DNA properties. The selected matrices included a TE-buffer, a silica nanoparticle solution (LUDOX HS-40), methylcellulose solutions (1% and 1.7% w/v) and alginate solutions (1% and 2% w/v) with 66 mM CaCl2·H2O (MW: 110.98g/mol). All reagents, except TE-Buffer (Invitrogen, USA), were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Synthetic single-stranded and double-stranded DNA was purchased from IDT (Coralville, IA); sequences are found in Table S1. Sequences were designed to minimize hairpins and are identical for single and double-stranded conformations as reverse complements were used to create the DNA duplexes. DNA was resuspended in Milli-Q® water and quantification was performed using a BioDrop Duo+ microvolume spectrophotometer (Biochrom Ltd., UK). DNA was subsequently aliquoted and diluted in Ultrapure Water to the desired concentration.

Matrix Preparation
Alginate solutions were made using alginic acid sodium salt from brown algae in Milli-Q® water. To ensure complete dissolution, stock solutions of alginic acid were mixed overnight prior to rheological analysis the following day. A 132 mM stock solution of CaCl2·H2O was made by dissolving 3.65 g into 250 mL of Milli-Q® water; dilutions were used to obtain the concentrations used in our study. Alginate and CaCl2 were mixed following a 4:1 (alginate:CaCl2) volume ratio. Methylcellulose solutions were made by heating a third of the required volume of Milli-Q® water to 80°C; methylcellulose was added to the hot water and agitated using a stir bar until evenly dispersed. The remaining volume of water was added as cold water; the resulting solution was cooled on ice for 30 minutes and subsequently agitated for another 30 minutes. We added 50 µL of synthetic DNA of different sizes (22, 78, 98 bp), concentrations (10, 25, 50, 100 ng/µL), and stranded conformations (double stranded and double stranded) into each sample, creating 24, 5 mL samples per matrix (2 conformations * 3 sizes * 4 concentrations). DNA-free controls were made by replacing the DNA volume with Milli-Q® water.

Rheology
Rheological characterization of the DNA-containing samples was performed through flow (rotational) and dynamic (oscillatory) measurements using a Discovery HR20 stress-controlled rheometer with an advanced Peltier and solvent trap system (TA Instruments, New Castle, DE). Details for each rheological test completed are outlined in Table 1. Flow sweeps were performed to obtain viscosity measurements while strain amplitude sweeps and frequency sweeps were conducted to probe the viscoelasticity, microstructure and flexibility of our materials. Creep-recoveries were performed to determine the thixotropy and recoverability of our materials (DNA-containing matrices). All tests were conducted at 22°C following a 20-second pre-shear treatment at 0.1 rad/s and 10 points per decade were collected. The gap was set to 54 µm for the cone plate geometry and 500 µm for the plate-plate geometry. A solvent trap filled with water was used to maintain humidity and reduce sample evaporation during runs.

<table>
<thead>
<tr>
<th>Type of matrix</th>
<th>Geometry</th>
<th>Flow sweep conditions</th>
<th>Strain amplitude sweep conditions</th>
<th>Frequency sweep conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE-Buffer pH 8 (Tris HCl and EDTA)</td>
<td>60 mm 2° CP</td>
<td>1 – 1200 s⁻¹</td>
<td>0.01%-100% Constant oscillation frequency 6.28 rad/s</td>
<td>0.05 rad/s – 150 rad/s Constant amplitude 1.5%</td>
</tr>
<tr>
<td>Silica Nanoparticle solution (LUDOX HS40)</td>
<td>60 mm 2° CP</td>
<td>1 – 1200 s⁻¹</td>
<td>0.01%-100% Constant oscillation frequency 6.28 rad/s</td>
<td>0.05 rad/s – 150 rad/s Constant amplitude 4% for ssDNA</td>
</tr>
</tbody>
</table>
Further experimentation was carried out using the matrices that displayed the greatest differences between the physical properties of DNA. To maintain consistency and reproducibility between samples, DNA-free controls were made daily alongside DNA-containing materials; consistent vortex times (two, 5-second intervals) were used. 1% w/v alginate and 1.7% w/v methylcellulose solutions were synthesized and rheologically characterized as described above. Flow sweeps were extended to lower shear rates (i.e., start at 0.0001 s\(^{-1}\) and end at 1200 s\(^{-1}\)) to observe the first Newtonian plateau and obtain the zero-shear rate viscosity. Creep-recoveries were replaced with strain amplitude sweeps conducted at 3.14 rad/s to determine whether there was greater differentiation between DNA properties at a lower angular frequency. Data were treated as described above; additionally, principal component analyses (PCA) were computed from viscosity measurements and moduli values from both oscillatory tests. Results were visualized using ordination diagrams.

**Data analysis**

Flow curves were plotted for each sample and overlayed according to TSD. Viscosity values were recorded for the following shear rates: 1, 10, 100, and 1000 s\(^{-1}\), representing very low, low, mid, and high shear rates, respectively. Boxplots were created for these values and plotted for each matrix – boxplots were coloured according to conformation, size, and concentration of DNA. Critical shear rates were recorded and plotted according to the physical properties of DNA. For amplitude and frequency sweeps, moduli (storage – \(G'\) and loss – \(G''\)) and the loss tangent (\(\tan\delta; G''/G'\)) were plotted for each sample. For the strain amplitude sweeps, only values above 0.05% strain were used, since values below this threshold had too low of a signal-to-noise ratio. If present, critical strain (\(\gamma_c\)) and modulus crossover values were recorded and plotted as boxplots. T-tests or Wilcoxon tests were performed on the data used to make the boxplots to determine if the three different DNA physical properties produced significantly different results. Correlation matrices were built for all quantitative variables.

**PART II: DNA EXTRACTS**

**Blood sample collection and deposition**

Whole bovine blood was obtained from Windcrest Meat Packers (Port Perry, ON). Acid dextrose anticoagulant A (ACD-A) was added in a concentration of 12.5% v/v to an amber Nalgene bottle prior to blood collection. ACD-A was made by dissolving 0.8550 g of dextrose anhydrous, 0.8448 g of sodium citric dextrose, and 0.3072 g of citric acid, all purchased from Sigma-Aldrich (Oakville, ON), to 62.5 mL of Milli-
Q® water. RNA extracted from bovine blood with and without anticoagulant has shown no significant differences in concentration or quality metrics over time, suggesting that nucleic acid extraction can be successfully performed using anticoagulated bovine blood\textsuperscript{49}; it has also demonstrated utility in other TSD experiments\textsuperscript{50,51}. Bovine blood appears to have superior erythrocyte properties than other blood analogues\textsuperscript{50,51}. However, there are still differences in fluid dynamics between bovine and human blood that might need to be considered\textsuperscript{52}.

50 µL of blood was deposited into 1.5 mL plastic microcentrifuge tubes. Tubes, with the lids open, were placed into three separate boxes with an ajar lid, protecting the sample from sunlight and ambient lighting, while allowing airflow for the natural desiccation of the blood. Samples were stored at one of three temperatures: room temperature (RT; 22 ± 1°C), refrigerated at 4°C (3.4 ± 1°C) or frozen at -20°C (-18.3 ± 1°C). Relative humidity (RH) varied according to outdoor temperature; values fluctuated depending on the time of the year (39.1 ± 11.6%). One time series was conducted for each temperature with blood from the same biological replicate (i.e., same cow). DNA extractions were completed approximately 30 minutes after blood collection (T\textsubscript{0}), one (T\textsubscript{1}), three (T\textsubscript{2}), six (T\textsubscript{3}), nine (T\textsubscript{4}), 12 (T\textsubscript{5}) and 19 (T\textsubscript{6}) months after deposition of the blood. Five replicates from each temperature condition were used for each timepoint (except for T\textsubscript{0}, where there was only a total of five), producing a final dataset of 95 DNA extracts.

**DNA extraction and quantification**

DNA was extracted from bloodstains using the QIAamp DNA Investigator Kit (QIAGEN, Germany) and following the protocol for *Isolation of Total DNA from Small Volumes of Blood or Saliva*\textsuperscript{53}. Following the suggestion for increased DNA yields outlined in the manufacturer's protocol, columns loaded with buffer ATE were incubated at room temperature for five minutes before the final centrifugation step. DNA was subsequently eluted from the spin columns using 50 µL of buffer ATE. Extracts were stored at -80°C until rheological analysis. DNA extracts were then thawed and 1µL was quantified on the BioDrop Duo+ prior to integration into the respective matrix for rheological testing. Smaller elution volumes were obtained for the 19-month samples which required pooling of replicates: 2, 3, and 2 samples, for the -20°C, 4°C and 22°C temperature conditions, respectively, were ultimately analyzed for this timepoint.

**Rheological testing of DNA extracts**

Findings supported the use of 1% w/v alginate with 66 mM CaCl\textsubscript{2} (Alg-Ca) as the matrix for DNA extract encapsulation (see Section 3 – Part I). A variety of experimental conditions were investigated to further optimize this matrix (Table 2). The concentration of CaCl\textsubscript{2} (33 mM vs. 66 mM), the reaction volume (750 µL vs. 3mL) and the wait time between the addition of CaCl\textsubscript{2} and loading (20 minutes vs. 60 minutes) were varied and compared. For both reaction volumes, we added 50 µL of DNA extract (i.e., one replicate extracted from a bloodstain) to each sample. In both cases, DNA was added to alginate prior to the addition of CaCl\textsubscript{2}. This amounted to 550 µL of 1% w/v alginate, 50 µL of DNA and 150 µL of CaCl\textsubscript{2} for the 750 µL samples, and 2.35 mL of 1% w/v alginate, 50 µL of DNA and 600 µL of CaCl\textsubscript{2} for the 3 mL samples. DNA-free controls were made and analyzed alongside extracts every day.

We performed both flow and dynamic oscillatory testing of DNA extracts in the 1% w/v Alg-Ca matrix (DNA extracts/alginate). Samples underwent the same pre-shear treatment as that used in the matrix optimization experiment and testing was conducted at 22°C. The solvent trap was filled with water and 10 points per decade were collected. All rheological tests were performed using a 40 mm plate-plate geometry and a gap of 500 µm. Flow sweeps were conducted from 0.0001 s\textsuperscript{-1} to 1200 s\textsuperscript{-1} for all samples, except for DNA extracts from bloodstains deposited 19 months. A gap assessment was carried out at three gaps (300 µm, 500 µm and 1010 µm) with DNA-free Alg-Ca matrices to evaluate potential wall slip during flow measurements. Strain amplitude sweeps were completed from 0.01% to 150% at a fixed angular frequency (\(\omega\)) of 6.28 rad/s. Frequency sweeps were performed from 150 rad/s to 0.005 rad/s at a constant oscillation strain of 2% (i.e., within the LVR). Experimental (instrumental or sample-related) limits are plotted in relevant figures: low-torque limit and secondary flow effects for flow sweeps, the low-torque limit for strain amplitude sweeps, and the low-torque limit and instrument inertia for frequency sweeps\textsuperscript{54}. Prior to the analysis of the 4°C extracts, a time sweep was carried out up to 8000 seconds at a constant angular frequency and oscillation strain of 6.28 rad/s and 2%, respectively. Follow-up dynamic oscillatory tests were performed with greater volumes of DNA; analyzed samples contained 50, 100, 200 or 400 µL of DNA, 550 µL of alginate and 150
µL of 66 mM CaCl₂. DNA in these samples were pooled extracts from freshly deposited bloodstains created from a different biological replicate (i.e., a different cow) than the one used during the time study. DNA was extracted as described above.

**Table 2: Experimental conditions for DNA extracts Alg-Ca according to bloodstain storage temperature.**

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>-20 °C (-18.3 ± 1°C)</th>
<th>4°C (3.4 ± 1°C)</th>
<th>Room Temperature (22 ± 1°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Timepoints</strong> (TSD; months)</td>
<td>- 1, 3, 6, 9, 12, 19</td>
<td>- 1, 3, 6, 9, 12, 19</td>
<td>- 0, 1, 3, 6, 9, 12, 19</td>
</tr>
<tr>
<td><strong>Replicates</strong></td>
<td>- 5 replicates/TSD</td>
<td>- 5 replicates/TSD</td>
<td>- 5 replicates/TSD</td>
</tr>
<tr>
<td></td>
<td>- Each replicate was used for 1 rheological test</td>
<td>- Each replicate was used for 1 rheological test</td>
<td>- Each replicate was used for 3 types of rheological test</td>
</tr>
<tr>
<td><strong>Rheological tests</strong></td>
<td>- 3 strain amplitude sweeps</td>
<td>- 2 strain amplitude sweeps</td>
<td>- 5 strain amplitude sweeps</td>
</tr>
<tr>
<td></td>
<td>- 1 flow sweep</td>
<td>- 1 flow sweep</td>
<td>- 5 flow sweeps</td>
</tr>
<tr>
<td></td>
<td>- 1 frequency sweep</td>
<td>- 2 frequency sweeps</td>
<td>- 5 frequency sweeps</td>
</tr>
<tr>
<td><strong>Reaction Volume</strong></td>
<td>750 µL</td>
<td>750 µL</td>
<td>3 mL</td>
</tr>
<tr>
<td><strong>Wait Times</strong></td>
<td>- 2 amplitude sweeps/TSD with 20 mins</td>
<td>- All tests were conducted after 60 mins</td>
<td>- All tests were conducted after 20 mins</td>
</tr>
<tr>
<td></td>
<td>- 1 amplitude sweep/TSD with 60 mins (except for 1-month replicate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 1 flow sweep/TSD with 20 mins</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>- 1 frequency sweep/TSD with 20 mins</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CaCl₂ concentration</strong></td>
<td>66 mM</td>
<td>66 mM</td>
<td>- 4 of 5 replicates/TSD were analyzed with 66 mM of CaCl₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 1 of 5 replicates/TSD was analyzed with 33 mM of CaCl₂</td>
</tr>
</tbody>
</table>

**Data analysis**

Data was treated and analyzed in the same manner described in the matrix optimization experiment. In addition, moduli values and tangent of the phase angle, tanδ, were either divided or subtracted by the control values for normalization and subtraction, respectively. Raw, subtracted, and divided values were plotted for each sample and grouped according to TSD. All rheological curves were plotted using OriginPro 2021b.

PCAs were performed from viscosity measurements, moduli (untransformed, normalized, subtracted), and loss tangent (tanδ) from both oscillatory tests. Top contributors from the original data to the new dimensions were recorded. The top principal components were plotted against time for each variable; correlation coefficients were recorded for each relationship. Linear models were built using principal components (PC1-PC3) as predictor variables and time as a response (dependent) variable. All statistical analyses were completed using R Version 4.0.3.
3. Results and Discussion

PART I: SINGLE DNA FRAGMENTS

The DNA-containing matrices demonstrated different rheological responses due to their physical and chemical properties (Fig. 1, Fig. S1). The gel-like matrices (alginate and methylcellulose) displayed shear-thinning behaviour while the silica and TE-buffer displayed Newtonian behaviour (Fig. 1A). The insertion of DNA into the silica solution and TE-buffer did not induce a non-Newtonian response to the increasing shear rates (Fig. 1). Although there appears to be shear-thickening behaviour in the TE-buffer (Fig. 1A), this is likely an artifact that can be attributed to secondary flow effects. Silica and TE-buffer with DNA occasionally displayed an LVE, however, viscoelastic moduli often fell below the limit of detection under large amplitude oscillatory shear (LAOS), indicating that DNA is not producing a strong enough response in these matrices (Fig. 1B).

Matrix optimization experiments concluded that the 1% w/v Alg-Ca was the best encapsulation matrix for DNA analysis. It produced the largest differentiation between the physical properties of incorporated synthetic DNA and yielded the strongest correlations between the physical properties of DNA and rheological parameters, especially during the follow-up optimization experiments (Fig. 2-3, Fig. S2). Alginate is a negatively charged linear polysaccharide composed of α-L-guluronic and β-D-mannuronic acid residues. A variety of soft gels and hydrogels can be formed via ionic crosslinking with divalent cations like Ca²⁺ and Mg²⁺; the carboxylate functional groups of guluronate residues coordinate with Ca²⁺, forming the well-documented “egg-box” model. Alginate-based materials displayed responses above minimum torque values and did not have any missing data in rheological tests, unlike the other aqueous matrices that were evaluated (Fig. 1, Fig. S1). This matrix also displayed the strongest trends relating to the physical properties of DNA, especially during the strain amplitude sweeps. The size of DNA influenced the strain and modulus values at the flow point (i.e., G’ = G”) during the first optimization experiment; specifically, larger DNA fragments required a greater amount of strain to flow than the shorter fragments and DNA-free controls (r = 0.45). The difference between flow points for the 22 bp and 98 bp DNA fragments was statistically significant (p-value = 0.003). In contrast, we found a negative association between the size of DNA and the modulus crossover (r = -0.38). Significant differences were observed between the 98 bp DNA and the shorter DNA fragments (p-value = 0.005 for 22 bp vs. 78 bp, p-value = 0.027 for 78 bp vs. 98 bp).

Fig 1: Rheological testing of different matrices containing 50 ng/µL of 78 bp double-stranded DNA. A) Viscosity values were obtained from flow sweeps conducted from 1 s⁻¹ to 1200 s⁻¹ at 22°C. The % in the legend represents % w/v. The black lines in the bottom left and right corner represents the low-torque limit and secondary flow limit of the rheometer, respectively, when using the 40 mm plate-plate geometry. The low-torque limit and the secondary flow limit for the 60 mm 2° cone-plate geometry are lower than the
40 mm plate-plate limits and are not shown in the figure. B) Strain amplitude sweep conducted at a constant angular frequency of 6.28 rad/s at 22°C. The filled and unfilled shapes correspond to the storage and loss moduli, respectively. The black and orange lines also represent the low-torque limit in this test when using the plate-plate and cone-plate geometry, respectively.

The shorter, more consistent vortex times used in the second optimization experiment influenced the rheological responses of our materials as the values of certain rheological parameters differed from those recorded in the first experiment. Data from both experiments were therefore analyzed individually. Interestingly, the correlation between DNA size and crossover modulus value at the flow point was very similar between experiments for 1% w/v Alg-Ca matrices (Experiment 1: \( r = 0.38 \) vs. Experiment 2: \( r = 0.37 \)). We further observed a negative association between the size of added DNA and the strain flow transition index (FTI) \( (r = -0.58) \). FTI is a measure of brittleness: it is calculated by dividing the stress at the flow point by the critical stress. It can also be obtained using strain values; in this scenario, a smaller FTI indicates that a material is brittle while a larger value represents a more durable/flexible material. In general, DNA-containing materials produced larger FTI values than DNA-free controls, supporting the hypothesis that Alg-Ca controls form stiff networks that are destabilized by the insertion of DNA (Fig. S3). Materials with larger fragments of DNA exhibited more brittle behaviour than those with shorter DNA fragments (Fig. S3). The conformation of incorporated DNA demonstrated significant differences \( (p\text{-value} < 0.05) \) between flow points during strain amplitude sweeps conducted at an angular frequency of 6.28 rad/s (Fig. 2, Fig. 3).

DsDNA required larger amounts of strain to flow than the ssDNA and Alg-Ca control materials (Fig. 2A). Opposite trends were observed for the crossover modulus (Pa); control materials displayed a larger modulus than the ssDNA and dsDNA (Fig. 2B). Solid-like materials typically demonstrate shorter LVEs and require less strain to flow than more liquid-like materials. This suggests that the dsDNA is destabilizing the ionic crosslinking (and subsequent gelation) process between alginate and calcium cations through electrostatic repulsion with alginate \( (r = 58) \) and/or competition for \( Ca^{2+} \) binding. DsDNA has a greater charge density than ssDNA \( (59) \), creating more electrostatic interactions with alginate; this appears to create a material with a weaker microstructure and greater liquid-like behaviour than samples with ssDNA or without DNA. PCA performed on the data from strain amplitude sweeps delineated DNA-free materials from those with DNA, confirming that the presence of DNA influenced the recorded rheological responses (Fig. 2C-D).

From a forensic perspective, we would expect to see changes in the size and conformation of DNA extracted from biological evidence due to enzymatic activity, hydrolysis and oxidative stress \( (33,34,36-38,41,42) \). Determination of an optimal matrix and best rheological tests are key to the applicability of this technique to biological fluid TSD.
Fig. 2. Boxplots for the A) strain values (%) and B) crossover modulus values at the flow point in the strain amplitude sweeps for 1% w/v Alg-Ca samples, separated by DNA conformation (i.e., 22, 78 and 98 bp DNA were binned according to their conformation). Note that control represents the samples without any DNA. For both A) and B), significant differences were observed between DNA conformations and controls. P-values for the computed t-tests are shown in the figures. C) and D) depict ordination diagrams of PCA performed on storage moduli (G') values, and loss moduli (G'') values, respectively. Data were obtained from 1% w/v Alg-Ca strain amplitude sweeps conducted at a constant angular frequency of 6.28 rad/s. Samples are coloured according to the presence or absence of DNA in the 1% w/v Alg-Ca matrix.
Fig 3. A) Strain amplitude sweep for a subset of DNA-containing, ionically crosslinked solutions of 1% w/v Alg-Ca. Tests were conducted at a constant angular frequency of 6.28 rad/s from 0.05% to 150% at 22°C. Filled and unfilled shapes represent the storage (G') and loss moduli (G''), respectively. B) Correlation matrix for the physical properties of DNA and rheological parameters obtained during strain amplitude sweeps performed on 1% w/v Alg-Ca. The correlation matrix was built using results from the second optimization experiment (i.e., follow-up optimization experiment). Pearson’s correlation coefficient, r, indicates the correlation between two variables; larger and darker circles represent stronger correlations.

PART II: DNA EXTRACTS

Part II-A: Volume of DNA

We evaluated the influence of DNA volumes on the rheological responses of DNA-containing materials. Dynamic oscillatory experiments were conducted with increasing volumes of ATE buffer and DNA, for controls and DNA-containing materials, respectively. An increased volume of buffer decreased the G'₀ in strain amplitude sweeps, indicative of weaker microstructure and lessened elastic behaviour (Fig. 4A). The addition of the same volume of buffered DNA further decreased the G'₀ of the Alg-Ca relative to the control (Fig. 4A), supporting the hypothesis that incorporation of DNA disrupts the ionic crosslinking between alginate and calcium by inhibiting the formation of as many crosslinks, producing weaker gels relative to DNA-free materials. The singular exception is for the 400 µL volume; DNA-containing materials displayed a greater G’ than controls. We suspect that the DNA might be outcompeting alginate for coordination with calcium cations, forming its own network and increasing the elasticity of our material. A decrease in G’ was also observed when increasing the volume of buffer in the frequency sweeps (Fig. 4B). Increasing the liquid component of the system decreased the elasticity and flexibility of the Alg-Ca; the incorporation of DNA slightly increased the G’ relative to the same volume of DNA-free control. The DNA might be increasing the flexibility of the Alg-Ca network by disrupting ionic crosslinking, allowing for easier rearrangements and better elastic energy-storing capability. As matrix optimization experiments were performed using 50 µL of DNA, and as it is a common elution volume in DNA extraction protocols, we selected this volume of DNA for the TSD study. However, the difference in moduli values between the materials with 100 µL of DNA and 100 µL of buffer supports the use of this volume of DNA in future studies.
Fig. 4: Dynamic oscillatory tests for 1% w/v Alg-Ca materials with increasing volumes of buffer solution or buffered DNA. A) Strain amplitude sweep performed at a constant angular frequency of 6.28 rad/s and B) frequency sweeps performed at a constant strain amplitude of 2%. The diagonal line in the bottom left corner of A) (barely visible) indicates the minimum torque limit of the instrument. The diagonal line in B) represents the inertial limit of the instrument. In both A) and B) the filled and unfilled shapes represent the storage modulus and loss modulus, respectively. Shapes outlined in black represent the G’ of DNA-containing materials.

Part II-B: TSD study

Concentration of DNA extracts decreased with longer bloodstain TSD for the 4°C and RT storage conditions but increased in the -20°C condition (Table S2). Likely, the DNA was present in greater amounts than recorded at early timepoints for the -20°C temperature condition, but was not detected due to lower extraction efficiencies and/or issues with the BioDrop.

Similar qualitative trends were observed for the strain amplitude sweeps of DNA-containing 1% w/v Alg-Ca materials. Samples displayed elastic behaviour (G’ > G”) at small amplitude oscillatory shear (SAOS), followed by strain-thinning in the G’ and weak strain overshoot in the G” (Type III) after surpassing a critical strain threshold (γc) (Fig. 5A,C, Fig. S4). Materials then displayed a flow point (i.e., G’ = G”), signifying the onset of predominantly viscous behaviour during LAOS (Fig. 5). Incorporation of DNA extracts into the 1% w/v Alg-Ca slightly influenced the value of these parameters, particularly in cases with a reaction volume of 750 µL. For the 4°C DNA extracts, we obtained a correlation of 0.54 and 0.62 between strain and modulus values at the flow point and TSD, respectively. When normalizing by the control analyzed the same day, correlations generally remained similar or decreased (e.g., r = 0.62 and -0.07 for the trends above). Normalization of data allowed for the control of intrinsic variability in Alg-Ca and of environmental factors influencing the raw data (i.e., temperature, humidity). We previously demonstrated good reproducibility in control responses during strain amplitude sweeps using 3 mL reaction volumes, fresh alginate and 20-minute wait times between CaCl2 addition and loading. However, the variability observed between control responses during the testing of DNA extracts supported the implementation of normalization and/or subtraction techniques, allowing accurate comparison between samples analyzed on different days.

For -20°C extracts, tanδ slightly increased within the LVE for bloodstain DNA samples until 19 months of deposition, at which point tanδ displayed values similar to those at early TSDs (Fig. 5B). An outlier (T2B) was detected and removed before further analysis. PCA for normalized tanδ values revealed weak time-dependent groupings. The first two principal components (PC1 and PC2) demonstrated weak associations with TSD, while PC3 displayed a moderate negative correlation with TSD (r = -0.57; Table 3). Most variability within normalized tanδ occurred at the onset of non-linearity (i.e., between the critical strain, flow...
point and the onset of the strain overshoot in the loss modulus) and within the non-linear range (Fig. 5A,B, Fig. S5A). The non-linear behaviour of a material under LAOS is often described as a network model, consisting of both segments and junctions. Junctions between segments can be created or lost (i.e., destroyed); in a material with a weak G⁻ overshoot, both parameters are positive, with the loss parameter exceeding the creation rate. When inserting DNA in the Alg-Ca system, we observed more viscous behaviour compared to our DNA-free controls; tanδ values from materials with DNA from early timepoints increased more rapidly between the critical strain and flow point compared to materials with DNA from later timepoints (Fig. 5). Additionally, greater G' values were observed within the LVR for DNA from older bloodstains, compared to those from earlier timepoints (Fig. 5A). This suggests that DNA at early timepoints disrupts the ionic crosslinking between alginate and calcium to a greater extent than DNA at later timepoints, decreasing the creation rate and leading to greater propagation of yielding. We would expect larger DNA fragments at early TSDs than later TSDs, which are likely more disruptive to the crosslinking process than shorter fragments expected at later timepoints. Larger fragments of synthetic DNA produced more brittle materials than shorter fragments or DNA-free controls during optimization experiments; this appears to be occurring herein for the -20°C extracts.

Prior to the analysis of the DNA extracted from bloodstains stored at 4°C, we performed a time sweep confirming that a one-hour wait time between CaCl₂ addition and loading provided a greater gel-like and stable material than a 20-minute wait time (Fig. S6). During strain amplitude sweeps of 4°C extracts, we observed a time-dependent decrease in the G₀ until the 1-year extracts; 12 and 19-month extracts displayed similar behaviour as the 1-month extracts (Fig. 5C,D, Fig. S5B). PCA revealed a positive correlation between PC1 and time (r = 0.52; Table 3). As in the -20°C extracts, the encapsulation of DNA typically resulted in larger tanδ values than controls, highlighting the disruptive effect of DNA on the gelation and ionic crosslinking of Alg-Ca. Weaker timewise trends were observed for 4°C extracts than -20°C; the combination of a stiffer Alg-Ca matrix and greater DNA degradation at later timepoints might explain this observation. The decrease in G' values in the LVR up to 9 months after deposition also suggests different degradation pathways than those observed at -20°C. We hypothesize that intra- and interstrand crosslinks produced in the 4°C DNA extracts due to oxidative damage and formation of abasic sites disrupt the Alg-Ca matrix to a greater extent than at -20°C. The crosslinking likely formed larger, more disruptive DNA molecules. As depurination and subsequent crosslinking reactions between the abasic sugar and amino group of the nitrogenous base on the opposite strand happen faster at higher temperatures, we expect a larger number of these occurrences at greater temperatures. As TSD progresses, continuous degradation breaks down DNA into smaller, structurally damaged fragments which are not as disruptive to the Alg-Ca matrix.

The room temperature extracts provided the weakest timewise trends (Fig. S4, S8C); this is likely a direct result of the larger reaction volume (3 mL). The contribution of DNA is lessened with the increased volume of alginate and CaCl₂, producing high variability between replicates with the same TSD. One replicate from the 1-year timepoint was removed from analyses due to a spurious result. PCA revealed a negative correlation between PC1 and TSD, an opposite trend than previously observed with lower reaction volumes and cooler temperatures (Fig. S4). Halving the concentration of CaCl₂ did not improve the rheological response of the DNA-containing material; data often fell below torque and inertial limits in dynamic oscillatory tests (Fig. S7).
**Fig. 5:** Results from strain amplitude sweeps performed on Alg-Ca materials containing DNA extracts from bloodstains aged at A)-B) -20 °C and C)-D) 4 °C. Moduli values are plotted in A) and C), while tanδ values are shown in B) and D). T1 through T6 represents the different TSDs: T1 indicates 1 month of deposition, while T6 represents 19 months of deposition. The letters A and B in the legends are used to indicate technical replicates (i.e., both T1A and T1B contained DNA extracts from bloodstains deposited for 1 month). Minimum torque limits are below the y-axis shown herein.

**Table 3:** Results of PCAs performed on data obtained from rheological testing of DNA extracts. Normalized tanδ was the variable selected to conduct each PCA. The strongest correlation between principal component (PC) and time is displayed for each temperature condition. Adjusted R² values are provided for the strongest linear models. Time and log-transformed time (natural logarithm) were regressed against the listed PC.

<table>
<thead>
<tr>
<th>Test</th>
<th>Storage Temperature (°C)</th>
<th>Strongest correlation (r) with time</th>
<th>Adjusted R² (Time/Log(Time))</th>
<th>Time</th>
<th>Log(time)</th>
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<td></td>
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<td>Estimate</td>
<td>Confidence Interval</td>
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<td><strong>Strain Amplitude Sweep</strong></td>
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<td></td>
<td>-20</td>
<td>PC3: -0.57</td>
<td>PC3: 0.24/0.30</td>
<td>-1.91</td>
<td>-4.16 - 0.34</td>
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<tr>
<td></td>
<td>4</td>
<td>PC1: 0.52</td>
<td>PC1: 0.20/0.39</td>
<td>0.68</td>
<td>-0.1 - 1.47</td>
</tr>
<tr>
<td></td>
<td>RT</td>
<td>PC1: -0.32</td>
<td>PC1: 0.06/0.06</td>
<td>-0.43</td>
<td>-0.98 - 0.13</td>
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<tr>
<td><strong>Frequency Sweep</strong></td>
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Frequency sweeps for all DNA-containing materials (Fig. 6A, Fig. S8) displayed frequency-dependent viscoelastic behaviour (G’>G’’), indicative of the formation of dynamic gel networks. Modulus crossovers were occasionally seen at high oscillation frequencies due to a decrease in G’, indicating potential structural breakdown. These high-frequency G’ breakdowns in our DNA-containing materials might be true material responses but variability and unpredictability in these occurrences suggest that instrument and sample inertia might be influencing the results, restricting the interpretation of high-frequency oscillatory data. Molecular weight distributions and conformation of polymers, including DNA, should influence the moduli and crossovers during frequency sweeps. The absence of crossovers at low frequencies in our frequency sweeps is likely a result of the Alg-Ca networking within the matrix used to analyze DNA. Goudoulas et al. did not observe a crossover in frequency sweeps for ss- and dsDNA in concentrated glycerol solutions.

The strongest trend observed between normalized data from frequency sweeps and TSD was for normalized tanδ; we observed a correlation of -0.44 between PC1 and TSD for the RT extracts (Table 3, Fig. S8). T0 and T1 extracts demonstrated lower tanδ relative to later timepoints, suggesting a more flexible soft gel when incorporating DNA extracts with large fragments (Fig. S8). Frequency sweeps performed on the 4°C extracts produced results dependent on the TSD (Fig. 6A). Increasing TSD slightly decreased tanδ values; materials with DNA from later timepoints formed more flexible gels than those with DNA from earlier timepoints (Fig. S8). However, as seen in the strain amplitude sweeps, the T5 and T6 samples behaved more similarly to the samples with DNA from earlier timepoints (Fig. 6). As TSD progresses up to 9 months after deposition, there is an increase in the disruption of the ionic crosslinking within the Alg-Ca matrix, increasing flexibility and G’ values of the material. As the degraded DNA from T5 and T6 form smaller and more rigid secondary structures than at earlier timepoints, there would likely be fewer DNA interactions with the Alg-Ca matrix, disrupting fewer crosslinks and making conformational alterations more difficult, reducing the flexibility of the material. PC2 from PCA performed on the 4°C extracts demonstrated a correlation of 0.42 with time (Table 3). Large variability was observed in the frequency sweeps for the -20°C extracts, especially in the tanδ. PC2 from the PCA produced a correlation of 0.43 with TSD (Table 3).
Fig. 6: Frequency sweeps performed on Alg-Ca materials containing DNA extracts from bloodstains aged at 4°C. A) Moduli values are plotted against angular frequency. The inset highlights the differences between TSDs at low angular frequency. The black line represents the inertial limit of the instrument. Moduli values were above the low-torque limit of the instrument (data below the y-axis shown herein). B) Tanδ values plotted against angular frequency; the inset highlights material response from 1 to 10 rad/s. Timepoints are coloured identically: technical replicates from the same timepoint are shaped differently, as shown in the legend.

Flow sweeps displayed Newtonian behaviour at low shear rates, and shear-thinning behaviour at increasing shear rates (Fig. 7A). The plateau observed at low shear rates approximates behaviour at rest; as the viscosities of our materials do not change significantly during this time, we concluded that our materials do not flow at rest. This behaviour is typical of polymeric solutions; as shear rate increases, the polymer chains extend and align themselves in the direction of flow, reducing interchain and intermolecular interactions, thereby decreasing the apparent viscosity. A gap assessment was performed to ensure that wall slip was not occurring; viscosities were similar between selected gaps, confirming that measurements were not influenced by slip (Fig. S9). For all flow sweeps, the main variability occurred within the first Newtonian plateau; slope values within the power law region were similar, except sample T5B, which was excluded from further analysis. TSD trends in flow sweeps were weak, but DNA-containing samples using 4°C extracts could be distinguished from controls using PCA (Fig. 7B). The smaller reaction volume used for analysis of the -20°C and 4°C extracts increased the contribution of DNA to the rheological response compared to the 3 mL reaction volume used for the RT extract analysis. Specific viscosity measurements at 0.01 s⁻¹ and 0.1 s⁻¹ were above 0, demonstrating that DNA was responsible for increased viscosity at low shear rates. Importantly, the most optimized experimental conditions were used to analyze the 4°C extracts; the differentiation between controls and DNA-containing materials highlights the influence of DNA on the bulk rheological response of the Alg-Ca matrix (Fig. 7B).

Fig. 7: A) Flow sweeps for 1% w/v Alg-Ca containing DNA from bloodstains aged at 4°C. Viscosity is coloured according to TSD. B) Ordination diagram resulting from PCA performed on viscosity values. Samples are coloured according to the presence of DNA (i.e., control vs. DNA).

We detected and quantified DNA from bloodstains deposited for 19 months. Our results herein corroborated previous studies where DNA fragments, some as large as 1600 bp, were detected and amplified up to 1-year after bloodstain deposition. The long-term detectability of DNA highlights its ex vivo stability and potential as a biomolecule of interest for bloodstain TSD studies. However, DNA is rarely selected as a target biomolecule; works either analyze specific mRNAs or simply observe the survivability of DNA in biological fluids. We observed moderate to strong correlations between rheological responses and TSD (r = 0.57 to r = 0.52). Modelling the best rheological responses demonstrated low R² due to small sample sizes. However, relationships between DNA degradation and TSD were similar to other works using...
untargeted approaches. Cossette and Stotesbury combined the untargeted analysis of DNA degradation from bloodstains with UV-VIS spectroscopy. Automated high-sensitivity gel-electrophoresis was used to analyze DNA; regressions built using DNA-specific variables demonstrated low model fits, similar to what we observed (i.e., low R²). However, both works demonstrated that combining DNA and UV-VIS metrics generated stronger TSD models. This supports the future implementation of a combinatorial approach; rheology could be paired with a spectroscopic technique to obtain data pertaining to both DNA and protein degradation.

4. Conclusion
We have demonstrated that it is possible to analyze small amounts (ng/µL) of short synthetic DNA and DNA extracts using rheology by encapsulating DNA in viscous, soft gel materials. By using rheology to probe DNA oligos and DNA extracts at low concentrations, we also addressed the clear research gap that exists in the field of DNA rheology. We first determined that a 1% w/v alginate solution with 66 mM CaCl₂ was the best matrix for small DNA oligo encapsulation and that rheological responses varied according to the physical properties (i.e., size, concentration, and conformation) of the incorporated synthetic DNA. DNA-containing materials also provided different rheological responses than DNA-free controls. Additionally, we developed a proof of concept, amplification-free and untargeted approach for the determination of bloodstain TSD. Bloodstains were deposited and stored at -20°C, 4°C and room temperature up to 1 year after deposition; extracts were inserted into the Alg-Ca matrix and probed using dynamic oscillatory tests and flow sweeps. Statistical analyses uncovered time-dependent trends in rheological parameters, providing insight into the naturally occurring degradation of genomic DNA. Moving forward, we believe that increasing the volume of encapsulated DNA, augmenting sample size, and coupling this technique with approaches targeting other blood biomolecules could increase the accuracy, precision, and applicability of this technique.
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