Dextranol: A better lyoprotectant

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Abstract

Dextranol, a reduced dextran, prevents damage to stored dry protein samples that unmodified dextran would otherwise cause. Lyoprotectants like the polysaccharide dextran are critical for preserving dried protein samples by forming rigid a glass that protects entrapped protein molecules. Stably dried proteins are important for maintaining critical information in clinical samples like blood serum. However, we found that dextran reacts with serum proteins during storage, producing high-molecular weight Amadori-product conjugates. These conjugates appeared in a matter of days or weeks when stored at elevated temperatures (37° or 45°C), but also appeared on a timescale of months when stored at room temperature. We synthesized a less reactive dextranol by reducing dextran's anomeric carbon from an aldehyde to an alcohol. Serum samples dried in a dextranol-based matrix protected the serum proteins from forming high-molecular weight conjugates. The levels of four cancer-related serum biomarkers (prostate specific antigen, neuropilin-1, osteopontin, and metalloproteinase 7) decreased, as measured by immunoassay, when serum samples were stored for one to two weeks in dextran-based matrix. Switching to a dextran-based lyoprotection matrix slightly reduced the damage to osteopontin and completely stopped any detectable damage during storage in the other three biomarkers when for a period of two weeks at 45°C. Dextranol offers a small and easy modification to dextran that significantly improves the molecule's function as a lyoprotectant by eliminating the potential for damaging protein-polysacharide conjugation.

Introduction

Room temperature protein preservation is important for food, biologics, purified protein products (e.g. research, cleaning, and industrial enzymes), and clinical samples (1–3). Preservation is required to avoid both chemical (e.g. oxidation) and physical forms of damage (e.g. aggregation) since any damage can reduce therapeutic function, enzymatic activity, flavor, and clinical information value. Many different varieties of excipients have been employed to prevent different forms of damage, one major category being

41 polysaccharides. Large uncharged polymers like polysaccharides protect proteins by molecular crowding, water
42 replacement, and glass formation (4–8).

43 Dextran has frequently been used as a polysaccharide lyoprotectant in dry protein formulations, mainly
44 due to its high glass transition temperature, which enables room temperature storage (4,9,10). As an inert
45 additive (11), dextran is particularly suitable to be used as a preservative in pharmaceutical products (12–17).
46 As a result, there have been numerous drugs on the market that contain dextran as a preservative (18,19),
47 including biologics (20,21).

48 Dextran has one drawback, discovered in the food science field in early 1990s; storage of proteins with
49 dextran in low-moisture conditions can facilitate formation of protein-dextran conjugates. (22–25). This
50 reaction has been well documented to occur with various proteins (26–30). Dextran is a branched D-glucose
51 polymer by α-1,6 linkages and α-1,3 linkages at branch points with a single reducing end and multiple non-
52 reducing ends. The conjugation is formed via a Mailard reaction between dextran's reducing end and protein's
53 primary amines (N-terminus and/or lysine side chains) leading via a Schiff base to the Amadori product. While
54 these conjugates are mostly shown to form at elevated temperatures (≥50°C) over a time period of days, there
55 are reports of protein-sugar conjugates forming even at lower temperatures, typically over longer time scales.
56 (31–36)

57 Dextran-protein conjugates are larger and more soluble than un-modified proteins. The size of dextrans,
58 like proteins, covers the low to high kilodalton range and thus, conjugation can easily double or triple the size
59 of the un-modified protein. Like pegylation, dextran conjugation also increases a protein's solubility (37) and
60 also makes it a better emulsifier (23,38–40). Conjugation of carbohydrates with proteins also causes acidification
61 (lowering of isoelectric point) by removing positive charges from lysine residues (41,42).

62 Dry storage is an attractive alternative to the typical cryogenic storage for stabilization of proteinaceous
63 biomarkers in clinical biofluid samples like blood serum. Many clinical samples are collected each year and
64 stored in archival biobanks for diagnostic and retrospective biomarker discovery research. The ten largest
65 biobanks collectively house approximately 35 million samples currently (43), stored in mechanical freezers or

66 liquid nitrogen dewars. Cryogenic storage has a number of drawbacks, which could be overcome by room
67 temperature storage of the dried samples; high cost, need to maintain the cold-chain for transportation of
68 samples, and damage to samples due to freeze/thaw are the main ones (44).

69 Isothermal vitrification of clinical biofluid samples can mitigate the shortcomings of cryogenic storage
70 by allowing storage and handling of samples at room temperature without significant loss of relevant chemical
71 information (2). Isothermal vitrification is achieved by mixing the samples with a lyoprotectant cocktail that
72 increases the glass transition temperature upon water removal to the point that samples form a glassy
73 amorphous solid at or near room temperature. The lyoprotectant matrix that we previously developed (2) uses
74 a mixture of dextran and trehalose with additional low-concentration excipients. The lyoprotectant solution is
75 electrospun into a fiber matrix that, when a biofluid sample is added, simultaneously absorbs the sample as it
76 dissolves and mixes with it. We designed this method to reach a spatially uniform concentration of
77 lyoprotectants in the sample without requiring mixing, which it detrimental to proteins. Overnight drying of
78 the sample-lyoprotectant mix results in a hard, glassy sample that can be stored at room temperature(2).

79 In our work to preserve clinical serum samples by isothermal vitrification, dextran is essential since it
80 gives the dried samples the high glass transition temperature needed to maintain stability at room temperature.
81 However, conjugation of biomarker proteins with lyoprotectants like dextran could be very detrimental to
82 downstream proteinaceous biomarkers analysis, especially when analytical techniques that are based on specific
83 binding (such as ELISA) are to be utilized to detect activity. In this work, we analyzed the damage to vitrified
84 human serum proteins caused by conjugation with dextran during dried state storage, and we found that
85 replacing dextran in our lyoprotectant formulation with a reduced dextran (dextranol) (**Fig 1**) stabilized serum
86 proteins more effectively during prolonged storage at room temperature as well as at elevated temperatures of
87 37, and 45˚C.

88

89 **Figure 1. Polysaccharide modification.** The cyclic and aldehyde-containing linear forms of the reducing end
90 of a polysaccharide (e.g. dextran) are in equilibrium. The linear form is able to undergo a Maillard reaction with

protein amine groups during prolonged storage or upon the addition of heat forming a glycated protein (Schiff base or Amadori Product). Reduction of the polysaccharide aldehyde to an alcohol prevents the Maillard reaction during long term storage of proteins with the polysaccharide.

Methods

General

In the experiments conducted here, we used trehalose dihydrate (≥99% purity, Ferro-Pfanstiehl Laboratories, Waukegan, IL), dextran (35-45kDa, Sigma: D1662-500G/lot: SLBT9984), and sodium borohydride (Alfa Aesar: 88983/lot:S09D008). We purchased the other chemicals from Sigma. Anonymized human blood samples were collected from volunteers through the University of Minnesota's (UMN) Tissue Procurement Facility (TPF) following a UMN Institutional Review Board (IRB) approved protocol (Study Number: 1011E92892). To separate serum, we allowed whole blood to clot for at least 30 minutes and then centrifuged it for 10 min at 2000 RCF. We carefully aspirated the serum (the supernatant) at room temperature and placed it into a new centrifuge tube, taking care not to disturb the cell layer or transfer any cells. We then aliquoted the serum into microcentrifuge tubes for use in experiments.

Dextranol synthesis

We synthesized dextranol (i.e. reduced dextran) from dextran (from *Leuconostoc mesenteroides* 35-45kDa, Sigma) using a protocol adapted from Paul et al.(45) First, we prepared a solution of 10% w/v dextran in purified water. To this, we added 10 times molar excess of sodium borohydride. We observed mild bubbling as we stirred the solution for 20 hours, after which, we adjusted the pH down to ~5 using concentrated HCl.

To remove unreacted sodium borohydride and the byproducts, borane and NaCl, we buffer exchanged the solution into water using either dialysis (Fisherbrand 12,000-14,000 MWCO) or spin concentration tubes

(Amicon concentrations 3,000 MWCO). Filtration in spin concentrators was very slow, with a flow rate of approximately 5-8 mL/hour at 4000 RCF. We repeated this process until small molecule contaminants were diluted out to less than 1%. Then, we removed water by lyophilization.

H_1-NMR

Dextran and dextranol product were each dissolved in DMSO-d_6 to approximate saturation. We spun tubes to remove the insoluble aggregates and added the supernatant (0.75 mL) to NMR tubes. We collected NMR spectra on Bruker 600 MHz NMR. Disappearance of anomeric proton peaks at 6.7 ppm, and 6.3 ppm, corresponding to the alpha, and beta stereoisomers of the anomeric center, demonstrated the complete reduction to the alcohol (46).

Production of the nonwoven lyoprotectant matrix by electrospinning

We electrospun fibers to form a dry porous matrix from a lyoprotectant cocktail. The primary components of the cocktail were dextran or dextranol and trehalose. In our previous work, we developed a new dextran-based matrix (V1EX) where five low concentration excipients, 1.5% glycerol (v/v), 1% polyethylene glycol (w/v), 0.1% Tween 20 (v/v), 0.3% gluconic acid (w/v), and 0.2% glucamine (w/v) were incorporated in to the trehalose-dextran cocktail prior to electrospinning. We found the inclusion of these excipients enhanced the stability of the test proteins when desiccated (2). Here we used the same matrix formulation, except where we replaced dextran with dextranol (as described below).

To prepare the lyoprotectant cocktail, we dissolved trehalose (0.4 g/mL) and either dextran or dextranol (1 g/mL) in a solution of low concentration excipients (at concentrations mentioned above). First, we added trehalose to the solution and stirred at 200 RPM for 45 minutes to dissolve it completely. Then, we added dextran or dextranol in three stages, following each with stirring to facilitate dissolution of the solids. We then stirred the mixture overnight (16 hours) at 200 RPM, and at 150 RPM the following day for three hours to

136 eliminate most of the bubbles that formed during mixing. Finally, we allowed the solution to rest for an
137 additional 12 hours at room temperature to ensure total dissolution. We stored the solution at 4°C when not
138 in use.

139 We electrospun the solution into microfibers across a voltage differential in a controlled environment.
140 We filled 1mL syringes with the lyoprotectant cocktail and affixed a stainless steel 18-gage 0.5" long blunt-end
141 needle. A multi-channel syringe-pump (NE-1600 multi-syringe pump; New Era Pump Systems, Farmingdale,
142 NY) extruded the solution at a flowrate of 0.03 mL/min. We maintained 50% relative humidity at room
143 temperature in an environment chamber (Electro-tech Systems, Inc., Glenside, PA). We placed the tip of the
144 needle 15 cm away from an aluminum target between which we applied a voltage differential of 15 kV. These
145 conditions were optimized to result in the most uniform electrospun fiber diameter production with optimum
146 inter-fiber distance in the matrix (optimized for capillary adsorption speed vs. dissolution rate) resulting in a
147 well-controlled matrix architecture. After spinning, we dried the matrix in a vacuum chamber overnight to
148 reduce the residual moisture content. We sealed and stored the electrospun matrix in a refrigerator (4°C) until
149 needed.

150 Isothermal vitrification and storage

151 To vitrify serum samples, we aliquoted 50 mg of electrospun fibers into round-bottom screw-top
152 cryogenic vials. To this we added 150 μL of serum. We dried the uncapped tubes in a vacuum chamber (at ≤
153 -85 kPa pressure) containing Dririte for 24 hours. After this period, we capped the tubes and stored them either
154 at room temperature, or in an incubator set to either 37°C or 45°C for accelerated aging/high-temperature
155 storage experiments. We also prepared biologically matched control samples of serum by freezing and storing
156 the aliquots (without using the lyoprotectant matrix) at -20°C.

157 To reconstitute the vitrified samples, we added 1.5 mL PBS (Phosphate Buffered Saline) to the dried
158 samples and incubated the tubes for 1 hour with gentle shaking, followed by gentle mixing by pipetting. This
159 resulted in a sample that was 10-fold diluted relative to the original serum. We did not reconstitute the vitrified

serum samples to their original volume because this produced a very viscous and difficult to handle solution (due to the presence of lyoprotectant sugars in the solution).

Differential scanning calorimetry

To determine glass transition temperature of vitrified samples, we collected differential scanning calorimetry (DSC) measurements on a TA Instruments (New Castle, Delaware) DSC Q1000 V9.9 Build 303. A 2-10 mg piece of vitrified serum in either dextran-based or dextranol-matrix was analyzed by ramping the temperature to 150°C at a rate of 10°C/minute after equilibrating at -60°C. Data was collected every second. We analyzed the data using a custom python script that identified the T_g (glass transition) as the maximum derivative heat flow of the smoothed data between 20 and 80°C and the T_{gonset}(glass transition onset) as the temperature where the heat flow deviated from the linear glass region 10% toward the linear liquid region (each region defined by a linear regression of the most linear 4°C window centered within 5°C lower (glassy region) or higher (liquid region) than the T_g.

Gel electrophoresis and staining

We carried out gel electrophoresis using Invitrogen NuPAGE and NativePAGE system (ThermoFisher Scientific, Waltham, MA). For serum analysis, we loaded the equivalent of 0.4 μL of serum (i.e. 4 μL of diluted serum) per well in a 10 well gel. We prepared samples either with NativePAGE buffer for native gel electrophoresis or with LDS sample buffer and the reducing agent for denatured samples, and with LDS sample buffer and the reducing agent for denatured and reduced samples. We boiled both the denatured as well as the denatured and reduced samples for 10 minutes prior to loading on the gel. We ran the gels for 75 minutes at a potential of 150 V.

For general protein stain, we stained native gels using the NativePAGE cathode buffer per manufacturer's instructions. We stained the denaturing gels for total protein using Imperial Protein Stain (ThermoFisher Scientific). We detected glycoproteins in gels using Pierce Glycoprotein Staining kit (Pierce #24562, lot#SK258276) per manufacturer's instructions.

ELISAs

We performed enzyme linked immunoassays (ELISAs) for osteopontin, MMP-7, neuropilin-1, or prostate specific antigen (PSA), following manufacturer's instructions. We used the following kits: Human Osteopontin ELISA Kit (Abcam, Cambridge, UK, #ab192143), Human Neuropilin-1 ELISA Kit (Abcam #ab227901), Human Total Prostate Specific Antigen ELISA Kit (Abcam #ab188388), and Quantikine ELISA Human Total MMP-7 (R&D Systems Minneapolis, MN, #DMP700). Plates were read in a Tecan Infinite 200 Pro M Nano plate reader at 450nm.

TCA precipitation

We carried out TCA precipitation by mixing equal parts of resuspended vitrified serum (or the frozen serum control, equivalently diluted after thawing) and 20% TCA (trichloroethanoic acid). After mixing 100 µL of each part, we incubated on ice for 15 minutes. Subsequently, we centrifuged 10 minutes at 10,000 RCF, photographed the tubes, and decanted the supernatant. The pellets were suspended in 400 µL saturated guanidine hydrochloride for protein quantification.

Protein quantification

Protein was quantified using BCA Protein assay (ThermoFisher Scientific). Standard curve was constructed using bovine serum albumin (BSA) standard. Samples from TCA precipitation were analyzed; both the "soluble" fraction and "precipitate". Assay was quantified using a Tecan Infinite 200 Pro M Nano plate reader at 562 nm.

Results

We found that when we used dextran-based lyoprotectant matrix to preserve blood serum by isothermal vitrification, serum began to show signs of damage over time. The damage was detected by the appearance of high-molecular weight smearing seen in gel electrophoresis. While freshly vitrified (and immediately

reconstituted) serum appears identical to fresh or frozen serum on gel electrophoresis, after sixteen weeks the smearing becomes very prominent (**Fig 2**), especially when the samples are stored at elevated temperatures. Individual protein bands are less detectable due to decreased intensity and smearing. While smearing is possibly worse in native protein gels (**Fig 2A**), denatured (**Fig 2B**), and denatured & reduced (**Fig 2C**) samples also show smearing. This indicates that the smearing is neither due to non-covalent nor disulfide cross-linked aggregated proteins alone. Non-covalent aggregates would be broken apart by the boiling in LDS (lithium dodecyl sulfate) sample buffer and thus would not be present in the denatured gels. Even if aggregates had disulfide cross-linking, these bonds would be broken by the reducing agent DTT (dithiothreitol) present in the denatured and reduced gel; yet the smearing remains. This indicates that the smearing is due to a large, non-disulfide, covalent modification of proteins.

Figure 2. **High molecular weight smearing in vitrified samples.** Human serum after 16 weeks of storage in the presence of dextran show high molecular weight smearing. Serum was either frozen or vitrified in dextran-based lyoprotectant matrix. Vitrified samples were either stored at room temperature (RT) or 37°C. Vitrified samples were reconstituted in PBS. Gel electrophoresis was carried out using (**A**) native conditions, (**B**) denaturing conditions, or (**C**) denatured & reducing conditions. High molecular weight smears are present in vitrified samples and more pronounced in samples stored at higher temperature. Smearing does not disappear upon denaturation or reduction, showing that it is not due only to denaturation, aggregation, or intramolecular disulfide bond formation, but is due to another covalent modification. This suggests dextran modification of at least some serum proteins.

High molecular weight smearing and glycosylation increased both with storage time and storage temperature and were accompanied by increased solubility. Smearing was faint after only 1 month of storage at room temperature, but increased when either storage temperature was increased to 37°C or storage time was extended to six months (**Fig 3A**). These effects were additive as after six months of storage at elevated temperature the smearing and decrease in individual protein band contrasts was significantly worse than storage either for six months at room temperature or for one month at elevated temperature. Concurrent with the increase in smearing was the increased glycoprotein staining in the high molecular weight region (**Fig 3B**). This

232 indicates that high-molecular weight bodies in the smear are becoming significantly glycosylated. We also
233 noticed that samples that had become more soluble and would not precipitate efficiently using a standard TCA
234 precipitation protocol (**S1 Fig, S1 Table**).

235 **Figure 3. High molecular weight smearing worsens over time and stains positive for glycosylation.**
236 Serum samples were vitrified in dextran-based lyoprotectant matrix and stored for 1 or 6 months at either room
237 temperature (RT) or 37°C. Samples were run on SDS-PAGE under denaturing/reducing conditions and stained
238 (**A**) for total protein or (**B**) for glycoprotein. Smearing worsens with higher temperature storage and with
239 increased storage time. Glycoprotein stain indicates high-molecular weight smears are glycosylated; suggesting
240 covalent attachment of dextran to proteins.

241 Non-reducing dextran, i.e. dextranol, based fibers were able to better preserve serum proteins during
242 extended storage. We reduced the reducing end of dextran from an aldehyde to a more inert alcohol and verified
243 the complete reaction by observing loss of anomeric proton peaks in H_1-NMR (**S2 Fig**). Dextranol had similar
244 glass-forming properties to dextran (**S3 Fig**). Whereas vitrifying serum in dextran-based lyoprotectant matrix
245 resulted in significant high-molecular weight smearing, serum vitrified in dextranol-based lyoprotectant matrix
246 did not smear (**Figs 4 and S4**). Samples that were frozen, freshly vitrified in dextran-based matrix or dextranol-
247 based matrix were all virtually indistinguishable from fresh, never frozen serum when analyzed by gel
248 electrophoresis under either native (**Fig 4A**) or denaturing conditions (**Fig 4C**). However, after 140 days of
249 storage at 37°C, vitrified samples in dextran-based matrix showed significant smearing, while dextranol-based
250 matrix samples were almost indistinguishable from frozen or fresh serum.

251 **Figure 4. Dextranol-based matrix preserves proteins and prevents smearing compared with**
252 **dextran-based matrix.** Serum samples were either fresh, frozen, or vitrified in either a dextran-based or
253 dextranol-based lyoprotectant matrix. Serum was analyzed (**A** and **C**) immediately after vitrification (Day 1) or
254 (**B** and **D**) after storage for 140 days at 37°C. Vitrified samples were reconstituted in PBS. Gel electrophoresis
255 was carried out under (**A** and **B**) native conditions and (**C** and **D**) denaturing/reducing conditions. After two

256 weeks, smearing is visible in samples preserved in dextran, but not in samples preserved in dextranol. Gels from
257 intermediate days in **S4 Fig.**

258

259 Dextranol also protected isothermally vitrified serum and BSA when stored at high temperatures (45°C).
260 The glass transition temperature of the vitrified serum in either dextran or dextranol-based matrix was 50-55°C
261 (**S3 Fig**). Therefore, in all storage experiments, the storage temperatures were kept below 50°C. Storage at
262 higher temperatures allowed us to simulate longer duration storage at room temperature. While decay reactions
263 do not uniformly scale with temperature, we expect a doubling in decay rates every 5-10°C increase (47). Thus
264 37°C storage corresponded to two to eight times faster aging while 45°C storage corresponded to four to twenty
265 times faster aging. BSA vitrified and stored in dextran-based matrix immediately after drying looked identical
266 to a frozen control, but after storage for 1-2 weeks at 45°C, the band from the main BSA monomer was highly
267 diminished, largely replaced by a high-molecular weight smear (**Fig 5A**). Dextranol-based matrix was able to
268 effectively eliminate this damage, with the sample stored at 45°C for two weeks looking indistinguishable from
269 the freshly vitrified sample and very similar to the frozen aliquot.

270

271 **Figure 5. Dextranol protects human serum proteins and BSA when stored at 45° C.** Coomassie stained
272 gel showing results of high temperature storage of (**A**) BSA (Bovine serum albumin) and (**B**) human serum.
273 Immediately after vitrification, samples preserved in dextran and dextranol-based lyoprotectant matrices look
274 similar to frozen sample (left). After 7 days (mid), and 14 days (right) BSA stored in dextran-based matrix is not
275 in in the major monomer band but is instead mostly in a high-molecular weight smear. BSA stored in dextranol-
276 based matrix still resembles frozen. Note, it is common to see higher molecular bands in SDS-PAGE of BSA
277 due to irreversible multimer formation.(48,49)

278

In addition to protecting BSA, the dextranol-based matrix also protected human serum proteins when isothermally vitrified and stored at higher temperature. Most protein bands became largely diminished when the sample was stored for one or two weeks in a dextran-based matrix (**Fig 5B**), and were replaced by a high-molecular weight smear, but storage in dextranol-based matrix protected the proteins. The serum proteins look indistinguishable from freshly vitrified serum after 2 weeks at 45°C, and looked very similar to frozen serum.

In addition to providing overall protection to high abundance serum proteins, and albumin specifically, we also found that dextranol-based lyoprotectant matrix provided much better protection than the dextran-based matrix for four individual proteinaceous biomarkers in the high-temperature stored serum samples. We selected 4 cancer biomarkers to be tested for stability. All four biomarkers that were examined (prostate specific antigen, neuropilin-1, osteopontin, and metalloproteinase 7) showed losses (as measured by ELISA) after one or two weeks of storage at 45°C (**Fig 6** and **S2 Table**). In dextranol-based matrix, PSA (**Fig 6A**) and neuropilin (**Fig 6B**) levels on day 1 were slightly reduced, potentially due to the drying process (by 7 and 8%, respectively), but were stable during seven or fourteen days of storage. MMP-7 levels (**Fig 6D**) in dextranol-based matrices remained slightly above the frozen control throughout this experiment. However, osteopontin levels in the dextranol-based matrices fell in a similar pattern to that seen in dextran-based fibers, although not quite to the same extent.

Figure 6. Serum biomarker levels are better retained after storage at 45°C when vitrified in dextranol than dextran. ELISA analysis of biomarker stability in vitrified human serum stored at high temperature (45°C), preserved in either dextran-based (orange) or dextranol-based matrix (green). Four biomarkers examined were (**A**) PSA (prostate specific antigen), (**B**) neuropilin 1, (**C**) osteopontin, and (**D**) MMP-7 (matrix metalloproteinase 7). Serum samples were analyzed immediately after drying (day 1), and one week and two weeks after drying and storage. Values are normalized to biomarker content in frozen control samples. Error bars are standard deviation of three replicates.

Discussion

Our results show that dextran is not a good lyoprotectant for proteinaceous biomarkers, as it leads to the formation of dextran-protein conjugates, especially in the dried state. Conjugation is likely due to a Maillard reaction forming an Amadori product between the primary amines of a protein (lysine residues and the N termini) and the reducing aldehydes of the dextran chain. While our experiments show this reaction happens to a large extent in a matter of days during storage at higher temperatures (37°C or 45°C), even during room temperature storage, the product is readily apparent after four months. The addition of dextran to proteins was determined to affect their physical properties like solubility as well as their immunogenicity (as seen by decreased reactivity with ELISA antibodies). Decreased immunogenicity results in less accurate immunoassays, which is a problem for detecting biomarkers in stored biospecimens. This damage is concerning since dextran is currently used as a lyoprotectant in a number of pharmaceutical formulations, including formulations for vaccines where decreased immunogenicity would cause reduce efficacy.

Despite the knowledge of the conjugation potential of dextran in food science, researchers in the pharmaceutics filed, in spite of observing protein damage in the presence of dextran, did not identify the mechanism of the damage. Lyophilization research that focused on therapeutic proteins established that formulations containing dextrans cause increase in protein size, increases solubility, and altered acidity, but this was interpreted as aggregation or damage, and not attributed to dextran-protein conjugation. Studies using size exclusion chromatography observe increased protein size after storage with dextran and speculate that this is caused by "soluble aggregation" (50–56). However, size exclusion chromatography cannot distinguish dextran conjugated proteins from dimeric or oligomeric protein "aggregates". Thus, the "soluble aggregates" reported may instead be dextran conjugates (potentially, in addition to protein self-aggregation). Similarly, studies that used dextran as a lyoprotectant that reported increased solubility (57) and acidity (58) of the preserved proteins could also have observed the effects of dextran conjugation. It's known that the degree of dextran modification varies between proteins (22,23). This is likely, in part, due to differences in the number and position of surface lysines.

Specifically, Yoshioka et al. characterize dextran mediated damage of lyopholized beta gamma globulin as denaturation and/or aggregation, yet their results are more consistent with dextran-protein conjugation than

330 simple denaturation or aggregation.(50) They conducted experiments with different sizes of dextrans (10kDa-
331 510kDa) and reported that at constant weight fraction in solution, smaller size dextrans (i.e. higher numbers of
332 reactive aldehydes ends per gram of dextran in the solution) caused more damage. They proposed that "the
333 effect of the molecular weight of dextran on the protein stability … could be explained in terms of the
334 parameters obtained by 1H-NMR such as T_{mc} [molecular mobility changing temperature]". A simpler
335 explanation is that over 50-fold variation in molarity of the reactive aldehyde groups, between the smallest and
336 the largest dextrans, is the main cause. Yoshioka et al. also observed that the increases in sizes of the damaged
337 protein as measured by size-exclusion chromatography correlated with the molecular weight of the dextran; a
338 result that cannot be explained by just denaturation and aggregation, but would be expected if the "damaged
339 proteins" they observed were in fact protein-dextran conjugates.

340 Qi and Heller, also found evidence for dextran modification of proteins without identifying it as such
341 (12). They report that in liquid state storage, dextran damaged therapeutic peptide insulinotropin. This damage
342 was different from that observed in the absence of dextran and could be prevented by adding certain excipients
343 into the solution (sodium metabisulfate or amino acids) that could react with dextran's aldehyde group.

344 Pikal et al. also found evidence for dextran-protein conjugation, which they characterized as soluble
345 "aggregates" (52,53). When they stored human growth hormone freeze-dried in dextran it became larger as
346 measured by size-exclusion chromatography. The increase in the size of the protein in the presence of dextran
347 was greater than when it was lyophilized in its absence or with excipients like glycine, mannitol, hydroxyethyl
348 starch, or trehalose. Interestingly, Pikal et al. suggest that the damage observed when the protein was stored
349 with lactose was due to protein-sugar conjugates formed via a Miallard reaction, yet they didn't propose the
350 same mechanism for dextran-mediated damage.

351 Other researchers in pharmaceutical field have looked at intentionally covalently attaching dextran to
352 drug molecules (59). This has been shown to have numerous effects on a drug's function. Some of the effects
353 are desired; such as improved on-target efficacy (60), reduced toxicity (61), and increased stability (62). However
354 others found detrimental effects from conjugating drugs with dextran, including decreased activity (62) and in

355 one case, a dextran drug conjugate (dextran-doxorubicin) failed in a phase one clinical trial due to higher
356 hepatotoxicity than unmodified doxorubicin (63).

357 We found that a small chemical modification, converting dextran to dextranol, eliminates this unwanted
358 reaction and better stabilizes human serum proteins during storage. The reduction of dextran's terminal
359 aldehyde to an alcohol, forming dextranol, ensures inertness. This relatively small chemical modification on a
360 large (~40 kDa) dextran monomer produced a new lyoprotectant that has all the desirable physical properties
361 of dextran without the potential for the detrimental Maillard reaction. Dextranol has been synthesized before
362 to enhance iron crystal growth and the properties of iron colloids (45,64). However, this communication is the
363 first report on the use of dextranol as an effective lyoprotectant.

364 Unlike dextran, dextranol-based lyoprotectant matrix we developed was able to protect serum proteins
365 during extended storage with little damage. After 140 days at 37°C or 14 days at 45°C serum proteins stored in
366 dextranol-based matrix showed little sign of degradation by gel electrophoresis, while proteins in dextran-based
367 matrix largely reacted with dextran, visible as high-molecular weight smearing on gels. ELISA analysis of four
368 selected biomarkers showed that detected levels of three of the four biomarkers did not decrease over storage
369 at 45°C when stabilized using the dextranol-based matrix, while levels of all four fell significantly when stored
370 using the dextran-based matrix. Even osteopontin, the one biomarker that did degrade during storage in the
371 dextranol-based matrix, did not lose activity as much as it did in the dextran-based matrix. It is yet unknown
372 what the mechanism of damage was in this case. Osteopontin is frequently cleaved into smaller form, often by
373 thrombin (65). If this were happening in the vitrified sample it may show up as loss of osteopontin since the
374 ELISA antibodies were developed against full-length osteopontin (66). While it is known that inherently
375 disordered proteins like osteopontin (67) are more prone to degradation intracellularly(68), it's not known if
376 that's the case extracellularly.

377 While intentional conjugation of proteins with dextran can have useful applications by improving
378 protein's solubility and heat stability (27) or as improved food emulsifiers (22), conjugation will inevitably
379 change the physical and chemical properties of the proteins, which can cause loss of function and loss of

detection in clinical assays. Other carbohydrates; including lactose, maltodextrin, glucose, and galactomannan; are also known to similarly react with proteins over extended storage times (31–34,69,70). Thus, the use of dextran, or other reducing carbohydrates, as lyoprotectant agents, especially for prolonged times at higher temperatures should be avoided. The modified sugar polymer dextranol is an attractive lyoprotectant since it provides the glass-forming protection of dextran while avoiding the damaging Maillard reaction.

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Supporting Information

S1 Figure. TCA precipitation of serum proteins stored 35 days in dextran-based matrix. Diminished pellet size shows increased solubility somewhat in the sample stored at room temperature (middle tube) and largely in the sample stored at 37°C (right tube) compared to frozen sample without matrix (left tube).

S1 Table. Vitrified serum stored 35 days in dextran-based matrix is not effectively precipitated by TCA.

	Whole[a]	Precipitated[b]
	mg/mL[c]	mg/mL[c]
Frozen[d]	59	48
Vitrified (RT)[e]	60	46
Vitrified (37° C)[f]	58	12

[a] Whole protein concentration after resuspension of vitrified samples and thawing of frozen samples. [b] Protein remaining following resuspension after TCA precipitation. [c] Undiluted serum protein concentration reported. [d] Serum frozen and stored at -20°C. [e] Serum vitrified in dextran-based matrix and stored at room temperature. [f] Serum vitrified in dextran-based matrix and stored at 37°C.

S2 Figure. H_1-NMR showing complete reaction and disappearance of protons attached to the anomeric carbon. (**A**) Proton signals at (6.74 and 6.30 ppm) indicate dextran aldehydes, while (**B**) loss of these signals shows that aldehydes were completely reduced to alcohols in dextranol.

S3 Figure. Tg and Tgon of serum preserved in either dextran and dextranol. DSC traces of serum samples preserved in either dextran (**A**) or dextranol (**B**) based matrix after 30 days of storage at room temperature. Solid blue line is DSC data, dashed green line is linear glass-transition fit, dashed red line is the

571 linear fit of liquid region (fit to grey-shaded region), dashed cyan line is the liner fit of the glassy region (fit to
572 green-shaded region). The vertical tan line marks the glass transition temperature (T_g) at 54.7°C (dextran, **A**)
573 and 53.9°C (dextranol, **B**). The vertical gold line marks the glass transition onset temperature (T_{gon}) at 53.2°C
574 (dextran, **A**) and 53.1°C (dextranol, **B**).

575

576 **S4 Figure. Dextranol preserves proteins and prevents smearing compared with dextran.** Serum samples
577 were either fresh, frozen, or vitrified in either a dextran-based or dextranol-based lyoprotectant matrix. Serum
578 was analyzed after 1, 7, 14, 28, 60, and 140 days at 37°C (data from days 1 and 140 in **Fig 4**). Vitrified samples
579 were reconstituted in PBS. Gel electrophoresis was carried out under both native conditions (**A**) and
580 denaturing/reducing (**B**) conditions. A duplicate denatured/reduced gel from the sixty day old sample was also
581 stained for glycoproteins (**C**). After two weeks, smearing is visible in samples preserved in dextran, but not in
582 samples preserved in dextranol.

583

584 **S2 Table. Biomarker preservation after 14 days storage of vitrified samples at 45°C[a]**

Fibers	Osteopontin	Neuropolin	MMP-7	PSA
Dextran[b]	61% ±1%	39% ±1%	82% ±4%	69% ±4%
Dextranol[c]	77% ±1%	87% ±3%	107% ±3%	94% ±3%

585 [a] Amount of indicated protein as a percentage of frozen sample without lyoprotectants, ± the standard deviation
586 of three technical replicates. [b] Serum vitrified in dextran-based matrix. [c] Serum vitrified in dextranol-based
587 matrix.

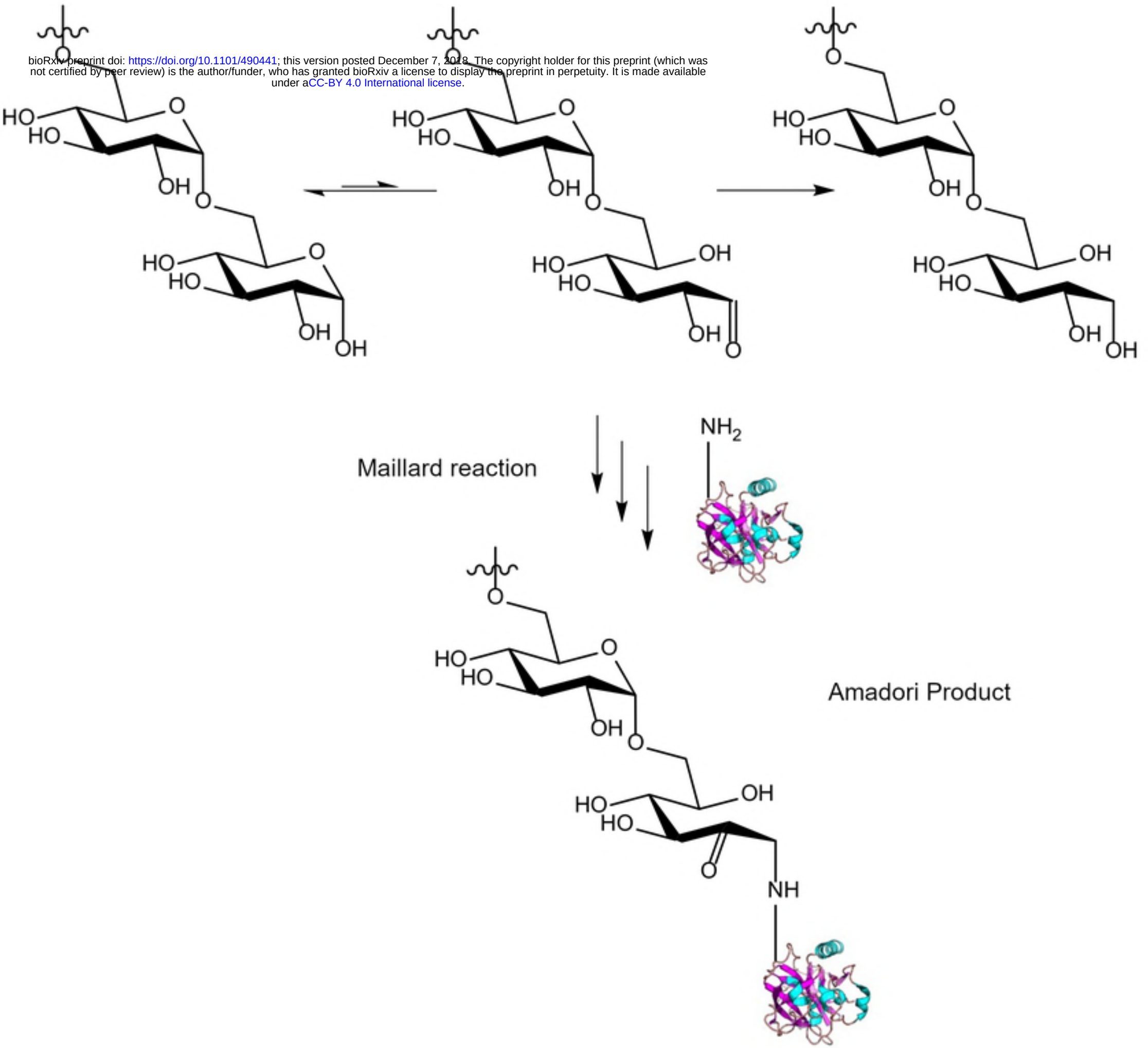


Figure 1

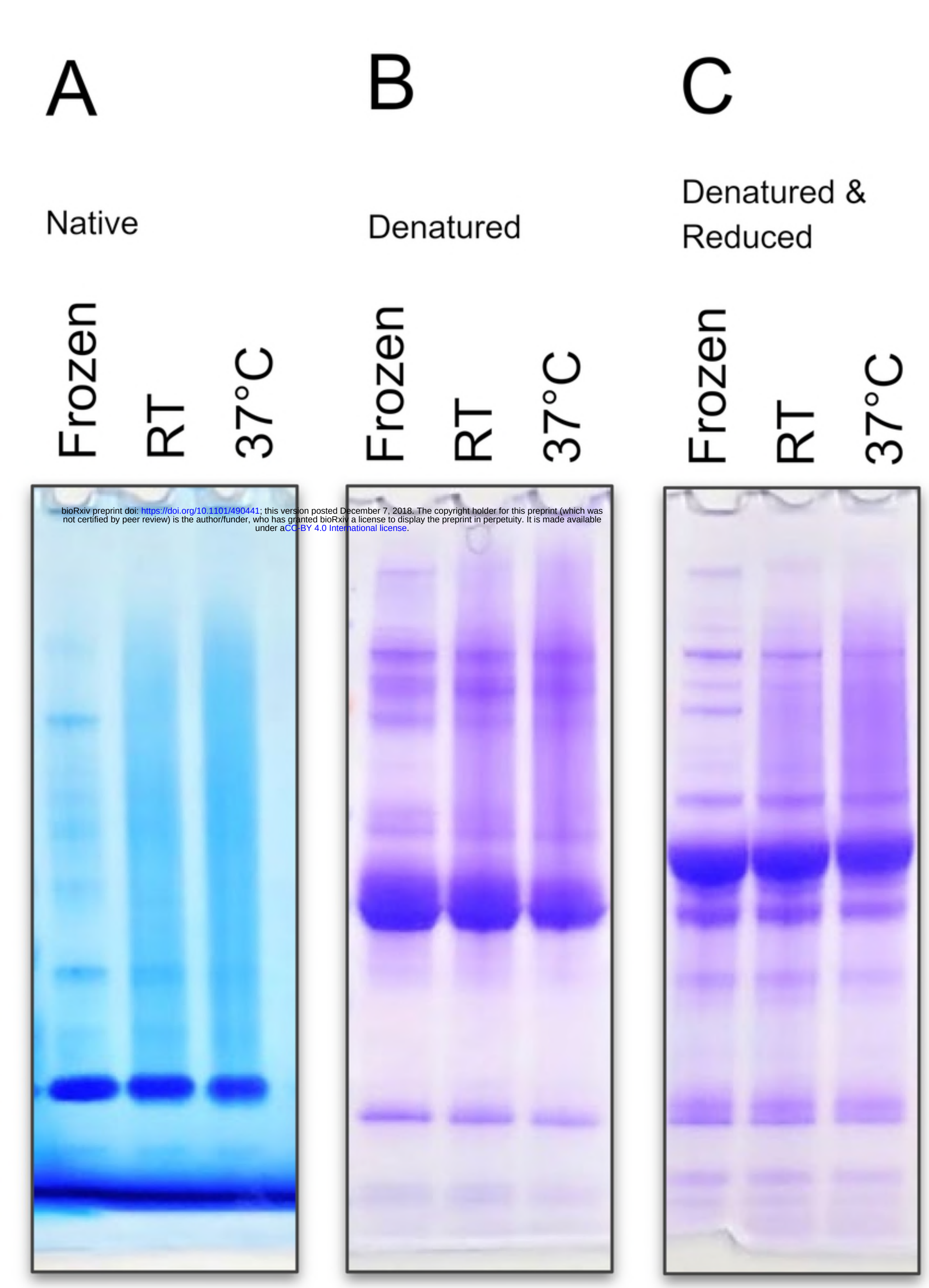


Figure 2

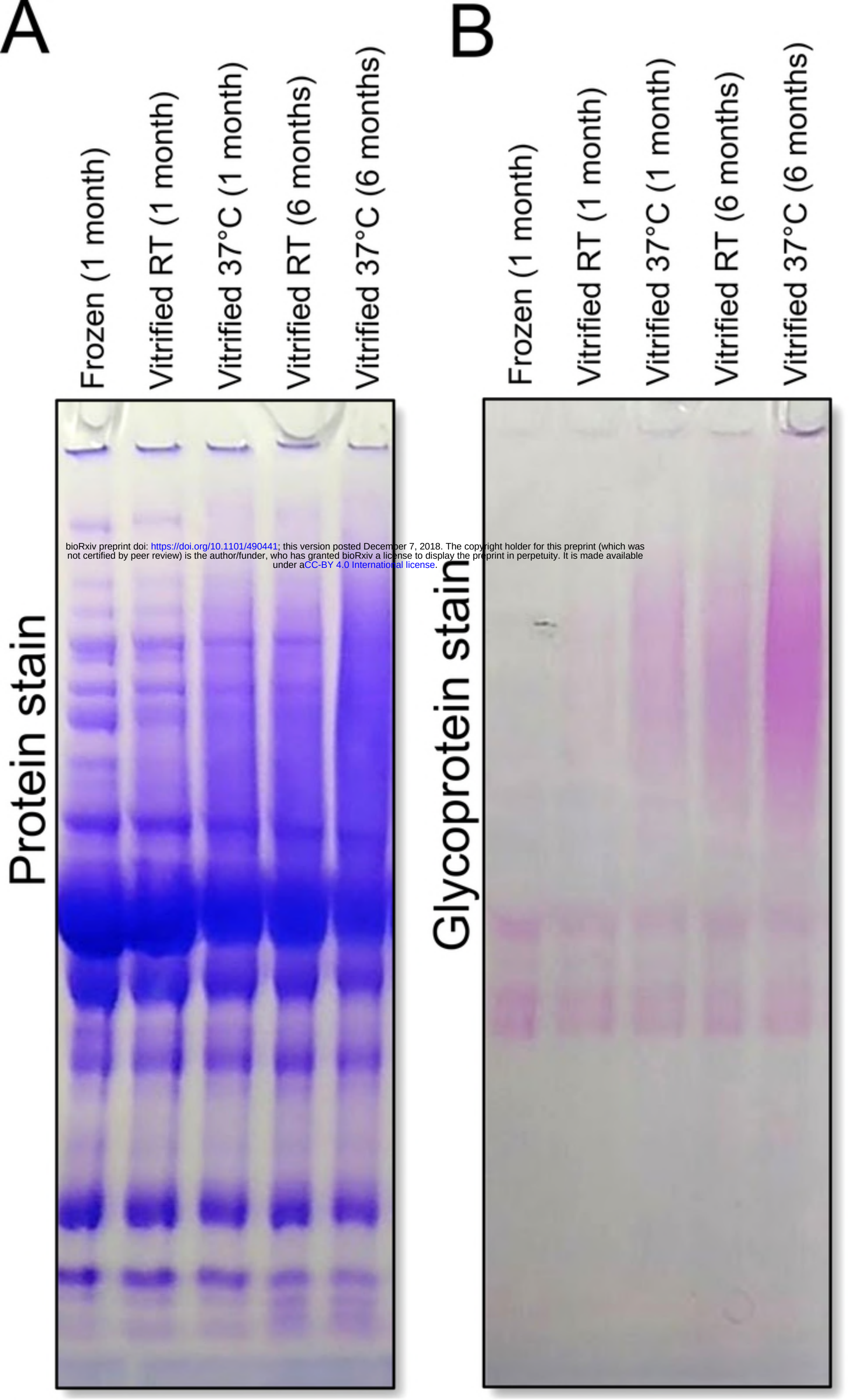
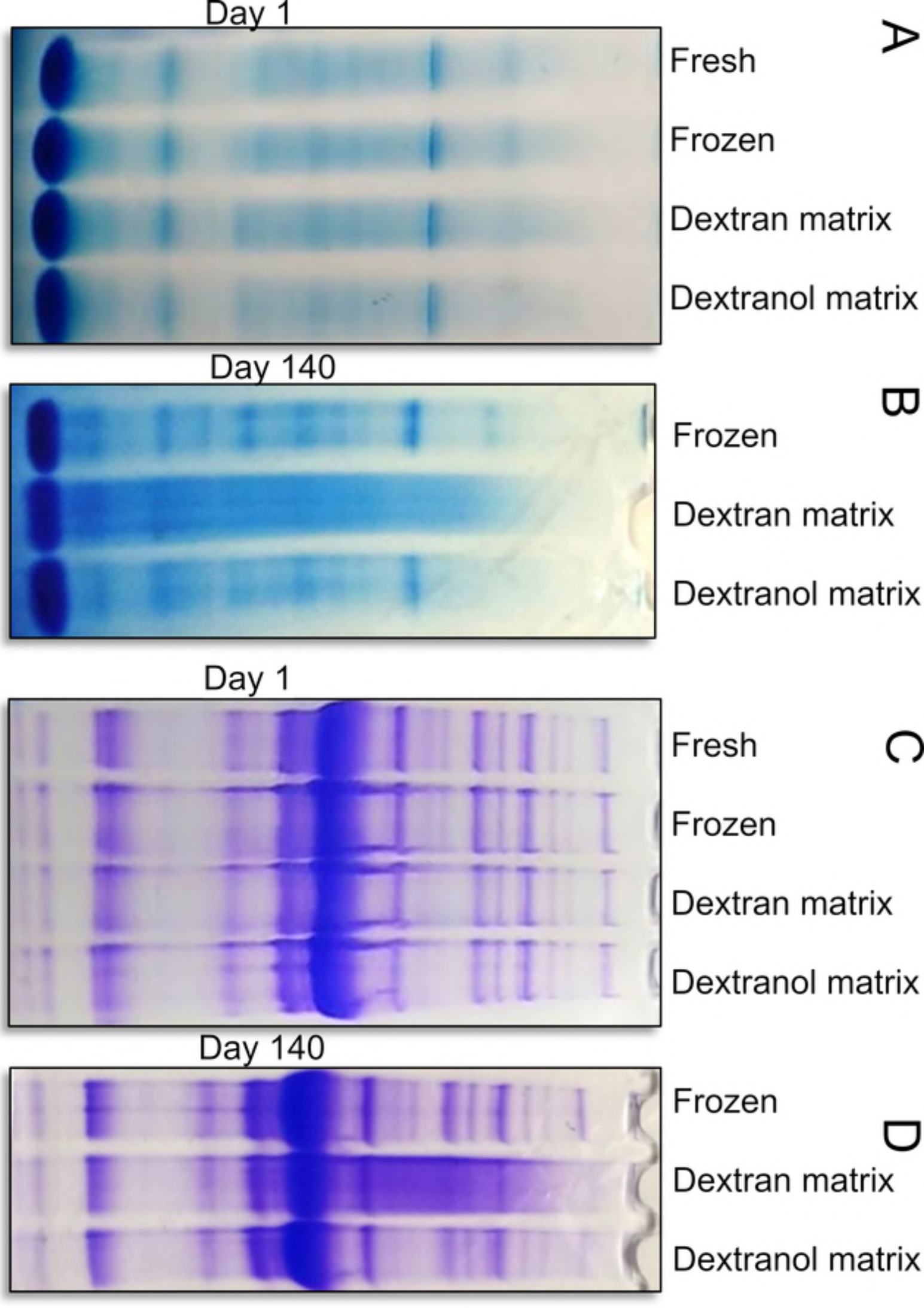


Figure 3

Figure 4


Day 1
A
Fresh
Frozen
Dextran matrix
Dextranol matrix
Day 140
B
Frozen
Dextran matrix
Dextranol matrix
Day 1
C
Fresh
Frozen
Dextran matrix
Dextranol matrix
Day 140
D
Frozen
Dextran matrix
Dextranol matrix

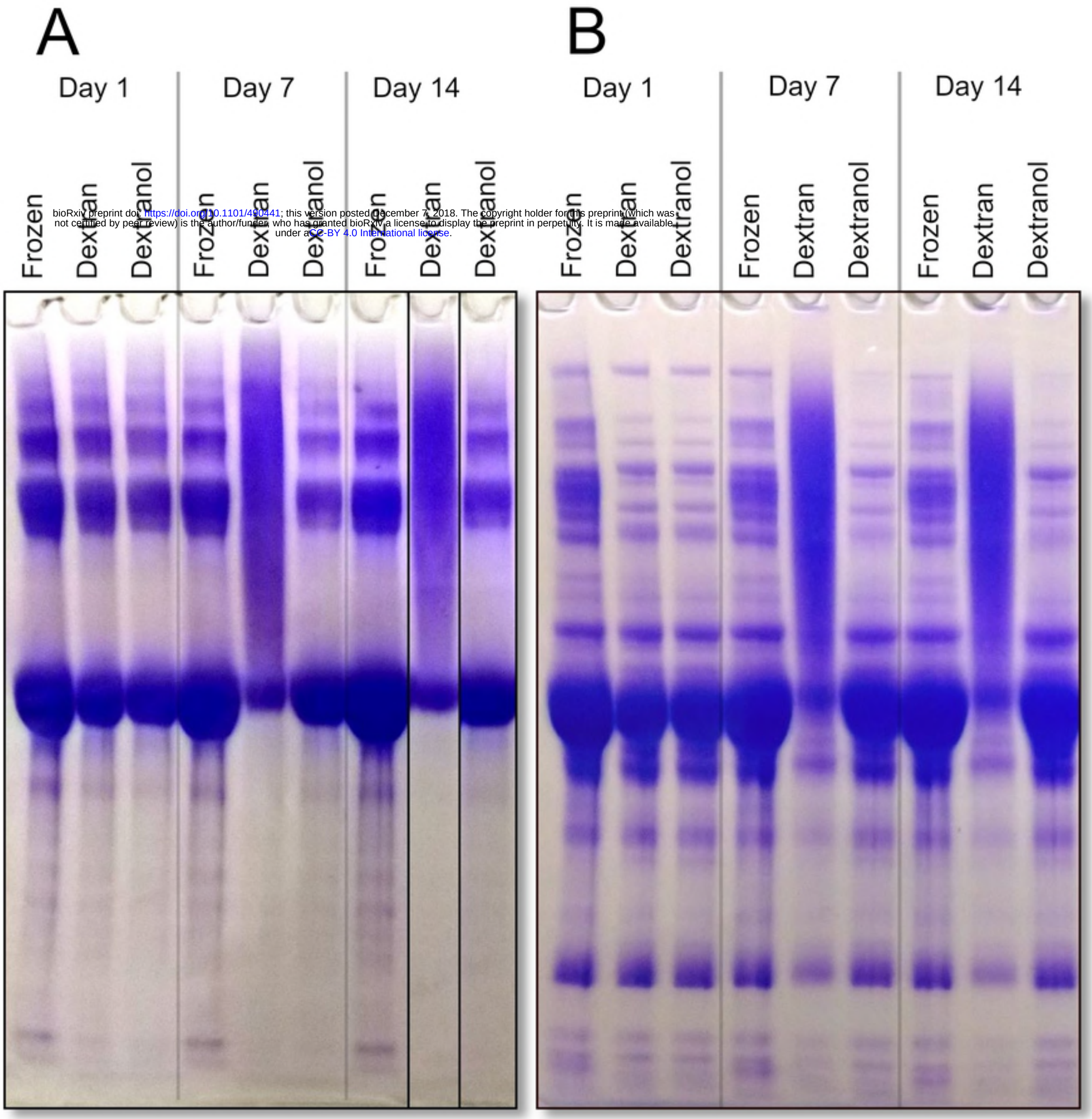


Figure 5

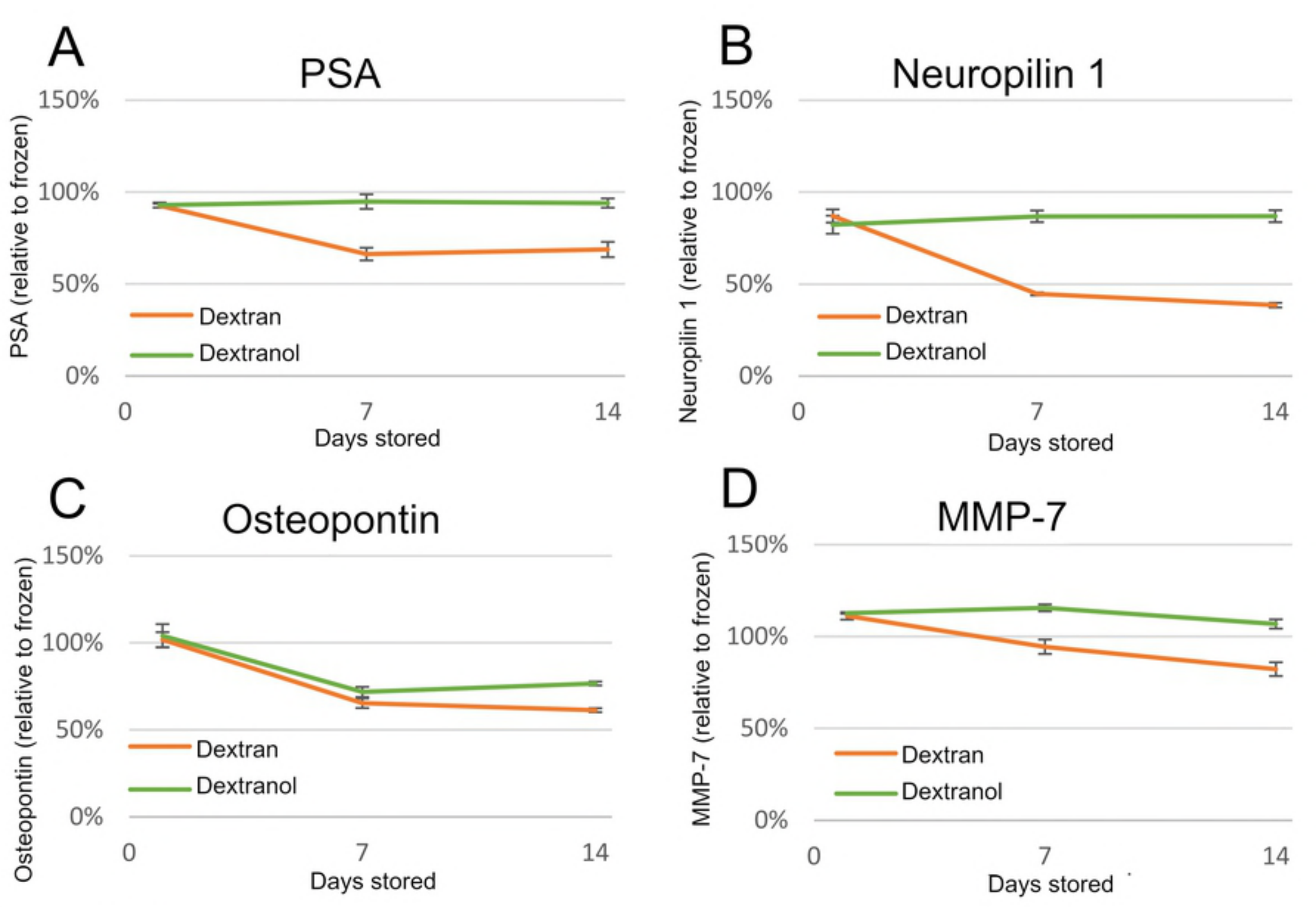

A
PSA
150%
100%
50%
0%
PSA (relative to frozen)
Dextran
Dextranol
0
7
14
Days stored
B
Neuropilin 1
150%
100%
50%
0%
Neuropilin 1 (relative to frozen)
Dextran
Dextranol
0
7
14
Days stored
C
Osteopontin
150%
100%
50%
0%
Osteopontin (relative to frozen)
Dextran
Dextranol
0
7
14
Days stored
D
MMP-7
150%
100%
50%
0%
MMP-7 (relative to frozen)
Dextran
Dextranol
0
7
14
Days stored

Figure 6