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Exposure to Buffer Solution Alters Tendon Hydration and Mechanics

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Abstract

A buffer solution is often used to maintain tissue hydration during mechanical testing. The most commonly used buffer solution is a physiological concentration of phosphate buffered saline (PBS); however, PBS increases the tissue's water content and decreases its tensile stiffness. In addition, solutes from the buffer can diffuse into the tissue and interact with its structure and mechanics. These bathing solution effects can confound the outcome and interpretation of mechanical tests. Potential bathing solution artifacts, including solute diffusion and the effect on mechanical properties, are not well understood. The objective of this study was to measure the effects of long-term exposure of rat tail tendon fascicles to several concentrations (0.9% to 25%) of NaCl, sucrose, polyethylene glycol (PEG), and SPEG (NaCl + PEG) solutions on water content, solute diffusion, and mechanical properties. We found that with an increase in solute concentration the apparent water content decreased for all solution types. Solutes diffused into the tissue for NaCl and sucrose, however, no solute diffusion was observed for PEG or SPEG. The mechanical properties changed for both of NaCl solutions, in particular after long-term (8 hr) incubation the modulus and equilibrium stress decreased compared to short-term (15 min) for 25% NaCl, and the cross sectional area increased for 0.9% NaCl. However, the mechanical properties were unchanged for both PEG and SPEG except for minor alterations in stress relaxation parameters. This study shows that NaCl and sucrose buffer solutions are not suitable for long-term mechanical tests. We therefore propose using PEG or SPEG as alternative buffer solutions that after long-term incubation can maintain tissue hydration without solute diffusion and produce a consistent mechanical response.

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Conflict of interest

The authors do not have any conflict of interests to disclose.

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Keywords

Tendon; Tendon Mechanics; Tension; Stress Relaxation; Buffer Solution

Introduction

A buffer solution is often used to maintain tissue hydration during mechanical testing; however, exposure to the buffer solution may alter the measured mechanical parameters, creating an artifact. The most commonly used buffer solution is a physiological concentration of phosphate buffered saline (PBS, 0.9% w/v = 0.15M). While the osmolarity of PBS corresponds to that of interstitial fluid, soaking tendon and other musculoskeletal tissues in PBS increases the water content (swells the tissue) which in turn decreases the tissue's tensile stiffness and/or modulus (Han et al., 2012; Screen et al., 2006, 2005). In addition to increased water content due to swelling, solutes from the buffer can diffuse into the tissue and interact with its structure and mechanics (Hannafin and Arnoczky, 1994; O'Hara et al., 1990; Travascio et al., 2009). These two bathing solution effects, change in hydration and solute diffusion, can have different time-dependence (Han et al., 2012; Hannafin and Arnoczky, 1994). Importantly, they can confound the outcome and interpretation of mechanical testing. This is a particular concern in longer duration tests such as stress relaxation or cyclic fatigue. Thus, it is important to consider the impact of altered hydration and solute diffusion when selecting a bathing solution, particularly for long duration experiments. When the study treatment is designed to alter the mechanics (for example, cause mechanical fatigue damage) the choice of bathing solution is even more important to prevent bath artifacts and to isolate the effect of mechanical loading treatment.

Many buffer solutions have been considered for mechanical testing, including small solutes (e.g., simple salts or carbohydrates), and larger molecules such as paraffin and polyethylene glycol (PEG). Ringer's solution, which is an NaCl-based isotonic solution, was used to keep human tendon hydrated during fatigue tests (Schechtman and Bader, 1997). Sucrose at a high concentration (10% w/v) was used to keep the apparent water content of bovine digital extensor tendons close to the level of fresh-frozen samples during overnight incubation prior to compression tests (Buckley et al., 2016). It is likely that these long term studies in salt or sucrose experienced solute diffusion into the sample. Saturated liquid paraffin was used during 56 hour fatigue tests of wallaby tail tendon (Wang et al., 1995), presumably maintaining water content. A mixture of 1.5% PEG-20 kDa and PBS was used to bathe sheep digital extensor tendons for one hour incubation followed by a 4.33 hour cyclic tensile test, where "it was assumed that the PEG's high molecular weight prevented their penetration into the tendon's interior" (Sverdlik and Lanir, 2002). PEG is a large hydrophilic polymer (Parsegian et al., 1986; Steuter, 1981), which, has the ability to maintain the tissue water content (Lujan et al., 2007b; Shahmirzadi and Hsieh, 2010). It has been suggested that high molecular weight PEG does not diffuse into the tissue (Katz and Li, 1973; Sverdlik and Lanir, 2002), but this has not yet been shown. Despite the numerous bathing solutions that have been used to regulate hydration, while some have measured the tissue apparent hydration, other bathing solution artifacts, including solute diffusion and the potential effect of the buffer on mechanical properties, are not well understood.

Therefore, the objective of this study was to measure the effects of long-term exposure to NaCl, sucrose, and PEG solutions on tissue hydration and mechanics, with the goal of maintaining hydration, preventing solute diffusion, and, most importantly, preserving the mechanical properties over several hours. Use of an appropriate buffer condition is necessary to ensure accurate measurement of tissue mechanics over long time periods.

Materials and methods

Tail tendon fascicles from 15 mature 8 month-old male Sprague-Dawley rats were used. Several fascicles were removed from each tail and randomly assigned to separate groups to study: 1) the change in water content after incubation in buffer solution (Hydration test), 2) the effect of solute diffusion on water content (De-/Re-Hydration test), and 3) the change in mechanical properties after short-term and long-term incubation in buffer solution (Mechanical test) (sample sizes shown in table 1).

Tails were harvested and stored at -20°C from animals used in a separate IACUC-approved study unrelated to tail tendons. Frozen tails were thawed in a water bath, and fascicles were harvested from a 60 mm long portion of the tail. Several solutes at a wide range of concentrations of NaCl, sucrose, and PEG were investigated (0.9%–25%, where % corresponds to % w, v_{solvent}) in order to achieve a range of tendon hydration (table 1). Based on the initial mechanical testing results, an additional solute group was added, SPEG, a combination of 0.9% NaCl and PEG. All of the solutions were made with Tris buffer (10 mM, pH 7.6) prepared with Tris base powder and hydrochloric acid solution.

Hydration test

To measure the change in fascicle water content after incubation in buffer solution, a Hydration test was used (figure 1A). Each specimen was incubated in a solution for 8 hr, which was selected to represent the time that might be needed for long duration mechanical tests. In addition, 8 hr is a sufficient to reach equilibrium weight, confirmed by preliminary studies (supplementary material figure S1). Following incubation, the sample was weighed (W_{wet}) (Mettler-Toledo AE240, Columbus, OH), dried in an oven at 43°C for 20 hr, and the final dry weight was measured ($W_{\text{dry},f}$). The Hydration apparent water content (ϕ_{app}^h) was calculated as

$$\phi_{\text{app}}^h = \frac{W_{\text{wet}} - W_{\text{dry},f}}{W_{\text{wet}}} \quad (1)$$

In addition, to match the hydration time used during the mechanical tests, some concentrations (0.9% NaCl, 25% NaCl, 8% PEG, and 8% SPEG), were incubated for only 15 min and the water content was measured. This was done because it takes ~15 min to prepare the sample for mechanical testing (clamping, measuring area, and preloading) and so it was important to quantify the hydration effects due to that short-term exposure to buffer solution.

De-/Re-Hydration test

Tendon is permeable to some buffer solutes, especially small solutes at high concentration (Hannafin and Arnoczky, 1994). In order to measure the effect of solute diffusion into tendon, which can lower the apparent water content by the inclusion of these diffused solutes in the 'dry weight' measurement, a De-/Re-Hydration test was performed (figure 1B), which excludes the diffused solute's weight and calculates the true water content (ϕ_{true}^r). Each sample was initially dehydrated and weighed ($W_{dry,i}$), rehydrated for 8 hr and weighed (W_{wet}), and dehydrated again and weighed ($W_{dry,f}$) (figure 1B). The solute diffusion into the sample is ($W_{dry,f} - W_{dry,i}$). The true water content (ϕ_{true}^r), which excludes solutes that have diffused into the sample, was calculated as

$$\phi_{true}^r = \frac{W_{wet} - W_{dry,f}}{W_{wet} - (W_{dry,f} - W_{dry,i})} \quad (2)$$

As in the Hydration test, the De-/Re-Hydration apparent water content (ϕ_{app}^r) was also calculated as

$$\phi_{app}^r = \frac{W_{wet} - W_{dry,f}}{W_{wet}} \quad (3)$$

If solute diffused into the sample, then ϕ_{true}^r would be higher than ϕ_{app}^r .

Mechanical test

The effect of buffer solution on the mechanical response of tendon was studied using a uniaxial tensile stress-relaxation test. Solutes and concentrations were selected for mechanical testing based on the results from the Hydration and De-/Re-Hydration tests. 0.9% NaCl (typical 0.15M used frequently) and solutions that closely maintained the fresh-frozen hydration (25% NaCl and 8% PEG and SPEG) (table 1). Sucrose was eliminated based on large amounts of solute diffusion into the sample.

Paired samples were obtained from each 60 mm fascicle by cutting the fascicle into equal proximal and distal portions and randomly assigned each portion to a short-term and long-term group. Randomization was performed to avoid bias due to variation in mechanical properties along the tail length (Danielsen and Andreassen, 1988). All mechanical tests were performed in a bath of 'treatment' buffer.

The tests were performed with an electromechanical uniaxial tester (Instron 5943, Norwood, MA). The cross sectional area (CSA) was measured immediately before beginning the mechanical test, with the tissue in the bath and under 0.01 N preload. Because rat tail tendon fascicles have a semi elliptical shape (Szczesny and Elliott, 2014), CSA was calculated for an ellipse using the width of the major and minor axes taken from images of each axes by rotating the sample 90 deg. The reference length (10 ± 0.8 mm) was also measured at a preload of 0.01N by using optically measured grip-to-grip distance. The mechanical test consisted of 5 cycles of preconditioning to 2.5% strain at 1%/sec, followed by a ramp to 5%

at 1%/sec, which was held constant for a 10 min stress relaxation. Force and displacement were measured throughout. Stress was calculated as force divided by the initial area and strain as displacement divided by the reference length.

Mechanical data analysis

The modulus (E) was calculated using the slope at the inflection point of the ramp portion of the stress-strain curve (figure 2A). The inflection point was determined from the first and second derivatives of a least-squares spline fit to the ramp data (Peloquin et al., 2016). For stress relaxation, a bi-exponential function was fit (figure 2B) (Anssari-Benam et al., 2015; Sverdluk and Lanir, 2002)

$$S(t) = S_0 - S_1 \left(1 - e^{-\frac{t}{\tau_1}}\right) - S_2 \left(1 - e^{-\frac{t}{\tau_2}}\right) \quad (4)$$

Where, S is the uniaxial stress, and S_0 is the peak stress, measured at the end of the ramp loading. Four relaxation fit parameters were: τ_1 and τ_2 , the relaxation time constants, and S_1 and S_2 , positive numbers that have the same units as stress. The equilibrium stress was calculated as $S_{eq} = S_0 - S_1 - S_2$. Curve fitting was performed in MATLAB.

Statistical analysis

For Hydration tests the average and standard deviation of ϕ_{app}^h were calculated for each solute and fresh-frozen fascicles, and a linear regression analysis was used to analyze the dependence of ϕ_{app}^h on buffer concentration. Also, the ϕ_{app}^h after 15 min incubation was compared to 8 hr incubation groups using two-tailed Student's t-test ($p < 0.05$). Each of the 15 min and 8 hr groups were compared to the fresh-frozen group using Dunnett's test ($p < 0.05$). For De-/Re-Hydration tests, a linear regression analysis was performed on both ϕ_{app}^r and ϕ_{true}^r followed by an analysis of covariance (ANCOVA) to compare the regression lines. In addition, for each solution ϕ_{true}^r was compared to ϕ_{app}^r using a two-tailed paired Student's t-test ($p < 0.05$). For the mechanical tests two-tailed paired Student's t-test was used to compare short-term and long-term groups (E , S_{eq} , CSA, τ_1 , τ_2 , S_1 , S_2) for each buffer ($p < 0.05$). Also, to test the hypothesis whether the short-term exposure changed the mechanics, a one-way ANOVA with a post-hoc Tukey's test was performed to compare across all buffers for the short-term groups ($p < 0.05$).

Results

Hydration test

The fresh-frozen apparent water content, ϕ_{app}^h , was $54\% \pm 5.6\%$ (figure 3). The ϕ_{app}^h decreased with an increase in solute concentration for all of the solution types ($p < 0.01$) (figure 3A, table 2). At the lowest concentration tested, 0.9%, all solutes over-hydrated the tissue compared to fresh-frozen: NaCl and SPEG over-hydrated the tissue a large amount, up to 66%, while the effect was lesser with sucrose and PEG, where hydration did not exceed 57% and 59%, respectively. Of interest is the concentration at which each solute matched the fresh-frozen ϕ_{app}^h (c_0 in table 2). The ϕ_{app}^h of NaCl and sucrose treatment groups were

above the fresh-frozen level until the highest concentration tested and matched fresh-frozen at 21.4% and 15.9%, respectively. The PEG and SPEG matched the fresh-frozen ϕ_{app}^h at 7.7% and 12.6%, respectively, and dehydrated the tissue at higher concentrations.

The difference between ϕ_{app}^h after 15 min and 8 hr incubation times was tested for a subset of concentrations. Only the water content of 0.9% NaCl at both incubation times and 25% NaCl at 15 min was different from fresh-frozen samples ($p < 0.05$) (figure 3B). There was no significant difference in ϕ_{app}^h between 15 min and 8 hr groups for 0.9% NaCl and 8% PEG solutions (figure 3B). However, for 25% NaCl, there was a significant increase from ϕ_{app}^h at 15 min ($44\% \pm 3.6\%$) to 8 hr ($52\% \pm 2.3\%$) ($p < 0.05$) at which time this concentration closely matched the fresh-frozen ϕ_{app}^h , and there was an increase for 8% SPEG, from ($50\% \pm 1.7\%$) after 15 min to ($58\% \pm 1.1\%$) after 8 hr of incubation ($p < 0.05$).

De-/Re-Hydration test

To measure the diffusion of solutes into the tissue, true (ϕ_{true}^f) and apparent (ϕ_{app}^f) water contents were determined using a De-/Re-Hydration protocol with separate samples from hydration test. The difference between ϕ_{true}^f and ϕ_{app}^f is an indication of diffusion of solutes into the tissue. The slopes of NaCl and sucrose solution for true and apparent water-contents were different ($p < 0.05$), while there was no difference between the dependence of ϕ_{true}^f and ϕ_{app}^f for either PEG or SPEG solutions (Table 3). When comparing the individual paired groups, there was no significant difference between the ϕ_{true}^f and ϕ_{app}^f for any solute at the lower, 5%, concentration (figure 4). The ϕ_{true}^f became greater than the ϕ_{app}^f for NaCl at 25% concentration ($p < 0.001$), and for sucrose at 15% ($p < 0.01$) and 25% ($p < 0.001$), indicating diffusion of solute into the sample. In contrast, there was no difference between the two water contents for PEG or SPEG at any concentration (figure 4), indicating there was no diffusion of solutes into the sample with these buffers.

Mechanical test

The modulus (E) of 0.9% NaCl was not different after short-term incubation (609 ± 240 MPa) compared to long-term group (463 ± 153 MPa) ($p = 0.06$), while for 25% NaCl, E decreased 24% from (1164 ± 253 MPa) to (887 ± 210 MPa) ($p < 0.01$) (figure 5A). In contrast, neither 8% PEG nor 8% SPEG changed E between short-term and long-term incubation. Following relaxation, the equilibrium stress of 0.9% NaCl showed a trending decrease between short-term and long-term groups ($p = 0.05$), however, for 25% NaCl, S_{eq} was significantly smaller after long-term incubation compared to short-term ($p < 0.01$), while there was no difference between short-term and long-term groups for either 8% PEG or 8% SPEG solutions (figure 5B). There was an increase in cross sectional area (CSA) for 0.9% NaCl, from (0.20 ± 0.08 mm²) to (0.23 ± 0.08 mm²) ($p < 0.05$) and a trending increase for 25% NaCl from (0.13 ± 0.04 mm²) to (0.14 ± 0.04 mm²) ($p = 0.05$), however there was no effect of incubation duration on CSA for either of the PEG or SPEG solutions (figure 5C).

The properties were compared across solutes for the short-term incubation and indicating short-term of exposure affected the mechanical properties and CSA ($p < 0.05$). In particular, E and S_{eq} were higher for 25% NaCl compared to 0.9% NaCl. Between 8% PEG and 8% SPEG, E and S_{eq} were not different. In addition, 0.9% NaCl had bigger CSA compared to

the rest of solutions. Across solutes E and S_{eq} had some varied differences between the NaCl, PEG, and SPEG concentrations (figure 5).

During the relaxation period, the two time constants were approximately an order of magnitude apart (figure 6A,B). The relaxation time constants did not change for NaCl solutions between the long and short incubation periods. However, the time constants for PEG and SPEG buffers had small changes in the long incubation period compared to the short incubation period: for PEG, τ_2 increased from 2.9 min to 3.3 min ($p<0.01$) and for SPEG τ_1 decreased from 7.4 sec to 6.7 sec ($p=0.05$) (figure 6A,B). There was a decrease in S_1 and S_2 for 0.9% NaCl, and 25% NaCl ($p<0.05$). For 8% PEG S_1 decreased ($p<0.05$), but S_2 was not different ($p=0.07$). However, for 8% SPEG there was no difference in S_1 and S_2 (figure 6C,D).

When comparing across solutions for the short-term incubation all the parameters had some level of variance ($p<0.05$). In particular, SPEG had a larger τ_1 than all other solutions, and PEG had a larger τ_2 than SPEG. There were no differences in time constants between the two NaCl concentrations. Indeed, S_1 and S_2 were larger for 25% NaCl compared to 0.9% NaCl, and for 8% SPEG compared to both 8% PEG and 0.9% NaCl solutions.

Discussion

The effect of different buffer solutions on hydration and mechanics of tendon was studied. We found that the conventional 0.9% saline solution is not a suitable choice for long duration mechanical tests because it does not preserve the hydration or the mechanical properties. The 8% PEG preserves the equilibrium mechanical properties during extended incubation times. We also showed that adding physiological concentration of NaCl to 8% PEG (SPEG) closely preserves the hydration equilibrium mechanics while creating a physiological ionic environment.

The incubation in NaCl solutions affected tendon modulus even during short-term exposure, likely due to rapid equilibration in this buffer (see Supplemental data). Our results for 0.9% NaCl between 15 min and 8 hr ($p=0.06$), did not indicate a significant difference in modulus. This finding is contrary to another study where the modulus of rat tail tendon decreased from 660 MPa when fresh-frozen to 390 MPa after overnight incubation in PBS (Screen et al., 2006). This difference in findings is likely due to the relatively large fresh-frozen modulus of 660 MPa in the later study, because the samples were kept hydrated during mechanical testing by spraying (Screen et al., 2006), whereas in our study the samples were in a bath, permitting swelling even during the short 15 min time period of the test. Similarly for ligament, exposure to 0.9% NaCl for 1 hr and 7 hr did not produce different stress-strain responses (Lujan et al., 2007b), likely due to the rapid equilibration in 0.9% NaCl (figure 3B). These results emphasize the time dependent effect of 0.9% NaCl solution on tendon mechanics and the rapid swelling rate for this solute.

The modulus in 25% NaCl was higher than in 0.9% NaCl, likely due to the prevention of increase in water content (swelling) (figure 3B). The modulus at hypertonic saline (e.g., 25% NaCl) has also been observed by others to be higher than physiological saline (0.9% NaCl)

and this difference attributed to be due to swelling and increased fibular spacing at the lower salt concentration (Han et al., 2012; Screen et al., 2006). For higher concentration 25% NaCl the amount of increase in CSA was not proportional to the decrease in modulus, thus area alone did not account for the differences. At 25% concentration diffusion of solute into the tissue occurred, where there is a difference between the true and apparent water contents (figure 4). This may have contributed in a decreased modulus after 8 hr of incubation in the bathing solution. It is also possible that there are other disruptive chemical effects associated with diffusion of solutes into the tissue (Hansen et al., 2009; Masic et al., 2015). Sucrose, as well, preserved the water content but had a significant diffusion of solutes into the sample (figure 3, 4). Our apparent water content with sucrose match previous results on rabbit MCL (Thornton et al., 2001), however, when excluding the effect of diffused solutes it is evident that even at high concentration of 25%, sucrose is not able to dehydrate the tissue.

It is likely that the initial dehydration step in the De-/Re-Hydration test may have affected the measurement of the true water content. Tissue water content after dehydration is not always recoverable (Meyer et al., 2013). Indeed, there was a small difference in the apparent water contents between the Hydration and De-/Re-Hydration tests. However, the trends with solute concentration are similar, therefore the comparison between the bathing solutions for the true water content (and the amount of solute diffusion) are accurate.

PEG and SPEG did not diffuse into tendon after 8 hr of incubation at any concentration (figure 4) which is in agreement with previous studies (Katz and Li, 1973). Additionally, neither 8% PEG nor 8% SPEG significantly changed the water content compared to fresh-frozen level (figure 3B). There was a small difference in water content of 8% SPEG between 15 min and 8 hr incubation times, which, although significant, was minimal. Similar concentration of SPEG buffer was used for tests on human ligament tissue, where 0.9% NaCl plus 7.5% PEG-10 kDa was used for mechanical tests (Lujan et al., 2009), and it was also shown to maintain the initial wet weight for 5 hr (Lujan et al., 2007a). For sheep digital tendon tensile test, 0.9% NaCl plus 1.5% PEG-20 kDa was used for mechanical testing (Sverdlík and Lanir, 2002). In the current study, we used PEG-20 kDa, which has large molecules and, consequently, a smaller chance of diffusion. Our pilot studies with PEG showed that although there is no evident diffusion after 8 hr, it is possible that PEG starts to diffuse into the tissue after 30 hr of incubation. For our samples the rate of diffusion is a particularly slow process in the scale of mechanical tests on tendon. Therefore, PEG is a solution that can be used for preserving initial water content and mechanical response of the tendon for at least 8 hours.

The 8% PEG bathing solution successfully maintained tendon modulus and CSA. However, it altered the transient behavior (figure 6), which may be related to imbalance between ions of the tissue and the buffer. That effect was eliminated by adding the physiological concentration of NaCl to PEG (SPEG), which suggests that there is an effect of ionic and non-ionic compounds in a buffer solution (Chahine et al., 2005). However, while SPEG maintained the slower τ_2 time constant, the faster τ_1 time constant was slightly reduced (figure 6A). This small difference of less than 1 sec in τ_1 for SPEG is minor and only influences the immediate transient behavior and will not affect the overall mechanical response (Reuvers et al., 2011; Szczesny et al., 2014; Thornton et al., 2001).

This study was performed using rat tail tendon, which is a simple highly aligned tendon that does not experience high loads compared to other tendons. This tissue is an excellent model system for the goal of this study. However the study outcomes, including, the initial water content, the buffer concentration where water content matches fresh values, and the diffusion rates, will vary with species, strain, tissue, sample size, and environmental conditions (Huang et al., 2011; Meyer et al., 2013; Urban and Maroudas, 1981). Here, our samples had slightly lower water content compared to the range of fresh tendon samples (Birch, 2007; McFarland et al., 1986; Screen et al., 2006). Other tendons (or ligaments) from other species will likely have different sensitivity to the environmental conditions (Rigozzi et al., 2013) and thus the concentration of PEG or SPEG should be specifically determined for any tissue of interest using a hydration curve (e.g., figure 3) for that specimen type. Another limitation is that we used grip strain, rather than optically measured strains, which could contribute to variability in mechanical measurements. Finally, this study was designed to maintain the “hydration” environment and mechanical properties and did not intend to replicate in vivo environment, where buffer solutes are just one of the several factors that are involved (Youngstrom and Barrett, 2016).

In conclusion, this study showed that NaCl buffer solutions are not suitable for long-term mechanical tests. Although several studies in the past have suggested alteration in tissue due to exposure to buffer solution, this study is novel in that 1) it takes a systematic approach for identifying the effects of buffer solution on hydration, 2) identifies the effect of solute diffusion into the sample (usually overlooked), and 3) guides the selection of the buffer and its concentration to optimally maintain both hydration and tendon mechanics for long-term testing. We propose using PEG or SPEG, a combination of normal saline and PEG, as alternative buffer solutions for tendon that after long-term incubation in the buffer solution can maintain hydration without solute diffusion and produce a consistent mechanical response, with the advantage of SPEG having a physiological ionic environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version.

Figure 1.

Schematics of Hydration and De-/Re-Hydration protocols. The last two steps are identical, however for the De-/Re-Hydration protocol the sample is first dehydrated so that the solute diffusion into the sample can be determined and the true water content calculated. W stands for weight and subscripts i and f stand for initial and final, respectively.

Figure 2.

Representative mechanical test data and analysis. (A) A spline was fit to the ramp portion of the data, and the modulus was calculated as the tangent at the first inflection point (green circle). (B) A bi-exponential function (red dashed) was fit to the relaxation data (black). The bi-exponential relaxation has two rates, fast (green dashed) and slow relaxation (blue dashed).

Figure 3.

(A) Hydration apparent water content and linear regression for each solute after 8 hr incubation. All solutes over-hydrate at lowest concentration (0.9%). Intercept with the fresh-frozen hydration (black lines) are 7.7% for PEG (red), 12.6% for SPEG (purple), 21.4% for NaCl (blue), and 16.0% for sucrose (yellow). Error bars indicate one SD and dashed lines indicate 95% CI for regression and fresh frozen lines. (B) Comparison between 8 hr and 15 min incubation groups showing time effect in 25% NaCl and 8% SPEG, and showing difference with fresh-frozen (FF) for 0.9% NaCl (15 min and 8 hr) and 25% NaCl (15 min). Error bars indicate one SD. * ($p < 0.05$) Compared 8 hr to 15 min, # ($p < 0.05$) compared to fresh frozen.

Figure 4.

True (solid symbol and line) and apparent (open symbol, dashed line) water content for different solutes for the De/Re-Hydration test. The difference between true and apparent water content for NaCl and sucrose indicates that the final dry mass was greater than initial dry mass due to solute diffusion into the sample. Error bars indicate (mean + SD) for true water content and (mean – SD) for apparent water contents. For reference, note that the mean fresh frozen water content was 54%. * ($p < 0.05$)

Figure 5.

Values for (A) modulus (E), (B) equilibrium stress (S_{eq}), and (C) cross sectional area (CSA) after short-term (15 min) and long-term (15 min + 8 hr) exposure to different solutions. * ($p < 0.05$), 'a' ($p = 0.05$), and 'b' ($p = 0.06$) between short and long term, and "—" ($p < 0.05$) between solutes at short term.

Figure 6.

Results from fitting of the relaxation curve. (A, C) Fast relaxation (τ_1 , S_1) and (B, D) slow relaxation (τ_2 , S_2) after short-term (15 min) and long-term (15 min + 8 hr) exposure to different solutions. * ($p < 0.05$) and 'b' ($p = 0.06$) between short and long term and "—" ($p < 0.05$) between solutes at short term.

Table 1

Specimen groups and sample sizes used for different tests.

	Solute	Concentration (%)	Incubation Time	Sample Size per Solute and Concentration
Fresh Frozen	N/A	N/A	N/A	97
Hydration	NaCl	0.9, 2, 5, 15, 25%	8 hr	5
	Sucrose	0.9, 2, 5, 15, 25%		
	PEG	0.9, 2, 5, 8, 10, 15, 25%		
	SPEG	0.9, 2, 5, 8, 10, 15, 25%		
	NaCl	0.9, 25%	15 min	
	PEG	8%		
	SPEG	8%		
De-/Re-Hydration	NaCl	5, 15, 25%	8 hr	5
	Sucrose	5, 15, 25%		
	PEG	5, 15, 25%		
	SPEG	5, 15, 25%		
Mechanical	NaCl	0.9%	8 hr and 15 min	17 pairs
	NaCl	25%		17 pairs
	PEG	8%		17 pairs
	SPEG	8%		17 pairs

Table 2

Hydration apparent water content linear regression to solution concentrations as $\phi = mc + b$, where c is the concentration of the bath. c_0 is the concentration that the linear regression intercepts with the average fresh-frozen water content, and CI is the 95% confidence interval of the intercept.

	m_{app}	b	c_0	CI	R^2
NaCl	-0.6 ± 0.1	66.7 ± 0.8	21.4	[18.6–25.4]	0.80
Sucrose	-0.2 ± 0.1	56.8 ± 0.9	15.9	[9.6–34.1]	0.28
PEG	-0.8 ± 0.1	59.9 ± 1.0	7.7	[6.0–9.3]	0.76
SPEG	-1.1 ± 0.1	67.7 ± 0.8	12.6	[11.6–13.6]	0.89

De-/Re-Hydration test linear regression to solution concentrations as $\varphi = mc + b$, where c is the concentration of the bath.

Table 3

	m_{true}	b_{true}	R^2	m_{true}	b_{true}	R^2
NaCl	$-0.5 \pm 0.1^*$	62.8 ± 1.0	0.85	$-0.7 \pm 0.1^*$	63.7 ± 0.8	0.95
Sucrose	$0.1 \pm 0.1^*$	54.3 ± 1.5	0.13	$-0.2 \pm 0.1^*$	56.2 ± 1.4	0.30
PEG	-0.5 ± 0.2	50.7 ± 2.9	0.37	-0.5 ± 0.2	51.5 ± 3.1	0.38
SPEG	-1.0 ± 0.1	64.0 ± 2.4	0.80	-1.0 ± 0.1	64.2 ± 2.4	0.80

* The slopes are different ($p<0.05$).