Statistical prediction of the vitrifiability and glass stability of multi-component cryoprotective agent solutions

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Abstract

Long-term biologic storage of articular cartilage has proven elusive due to cellular degradation over time or acute damage during attempts at cryopreservation. Vitrification is one option that may result in successful cryopreservation but difficulty with cryoprotective agent (CPA) toxicity at high concentrations of a single cryoprotectant has hindered development of successful protocols. This study was designed to determine the vitrifyability and glass stability of solutions containing combinations of commonly used CPAs and to document CPA interactions that occur. One hundred and sixty-four multi-CPA combination solutions of 6-9M were evaluated for vitrifyability and glass stability using direct visualization after immersion in liquid nitrogen for 30 minutes and upon warming. Binary and ordinal logistic regression analysis was used to statistically analyze each CPA for its ability to vitrify and its effect on glass stability in multi-component CPA solutions. Propylene glycol has the greatest incremental contribution to vitrification while formamide had the least contribution. A threshold was established whereby the ability of a solution to vitrify could be determined by calculation. Glass stability was not as clearly defined due to variability in the results; however, contributions of interactions between CPAs to the glass stability of solutions were determined. This study provided values that predict if a solution will vitrify. Furthermore, the glass stability of solutions containing multiple CPAs do not behave as linear additions of binary solutions and interactions between CPAs have a significant effect on the glass stability of these solutions. These variables should be considered when designing vitrification solutions.

Key Words: vitrification, glass stability, cryoprotective agents, logistic regression, articular cartilage, dimethyl sulphoxide, propylene glycol, glycerol, ethylene glycol, formamide

Introduction

There is significant medical need for replacement tissue and organs, whether from donors or tissue engineering techniques[24; 27]. Long-term biological storage with maintenance of cellular viability and tissue function has proven elusive[17], due to cellular degradation over time when stored at low supra-zero temperatures[32] or acute damage during attempts at cryopreservation[22; 23]. In articular cartilage, this acute damage during cryopreservation results from ice formation in the cartilage matrix when low concentrations of cryoprotective agents (CPA) are present[23] and due to toxicity when high concentrations of cryoprotective agents are used to prevent ice formation[14]. Ideally, solutions would vitrify, avoiding ice formation, without containing any CPAs; however, this would require cooling and warming the tissue at rates not possible in larger tissues (>10^6 K/s)[31]. Thermal conductivity of physiological solutions limits cooling rates to 10^2 K/min in volumes sufficient to contain transplantable tissue such as articular cartilage, decreasing towards 60 K/min as CPA concentrations are increased[23]. The variation in achievable cooling rates with CPA concentration is likely related to differences in convective currents as viscosity increases. It is clear that vitrification without the presence of CPAs is not an option, so CPAs must be used.

The use of multiple CPAs within a solution can result in decreased toxicity compared to solutions with a single CPA[17], but development of appropriate combinations that prevent ice formation while limiting toxicity requires an understanding of the solutions' vitrifiability and glass stability. Vitrifiability is the tendency for a solution to form an amorphous solid upon cooling and is often measured by calculating the critical cooling rate of solutions (rate that avoids ice formation)[25; 26; 29]. Glass stability is the resistance to devitrification upon re-warming and is often measured by calculating the critical warming rate of solutions. Vitrifiability and glass stability parameters have been measured for many CPAs[1; 2; 3; 4; 5; 6; 7; 8; 9; 10; 11; 12; 13; 19; 20; 28], but these have focused on milligram quantities of aqueous solutions with only one or two CPAs. The properties of solutions in large quantities can be significantly different[16], and solutions with more than two CPAs need to be tested in order to understand how these more complex solutions behave. An important obstacle is that the gold standard for the assessment of solution properties is to create a phase diagram, but phase diagrams require significant data collection and a new phase diagram must be created whenever a solution with different constituents is investigated[25; 26]. The more appropriate alternative is to use approaches requiring fewer resources (labour and physical resources) to survey available options and then use more detailed approaches (such as the building of phase diagrams) for solutions of particular interest[26].

This study investigated the vitrifiability and glass stability of 164 solutions composed of 3-5 CPAs in bulk volumes, evaluating the solutions optically. Multiple logistic regression was used to analyze the data collected. It was hypothesized that this statistical technique would allow the estimation of the contributions of individual CPAs to the vitrifiability and glass stability of solutions containing multiple CPAs.

Materials and experimental methods

One hundred and sixty four solutions of cryoprotective agents in 1x Dulbecco's phosphate buffered saline (pH= 7.0)(DPBS, Gibco, BRL, MD) were prepared using dimethyl sulphoxide (range of 0-4 mol/L concentrations) and propylene glycol, ethylene glycol, glycerol and formamide (range of 0-3 mol/L concentrations for each). Each solution contained a total of 6-9 mol/L of CPA. CPAs were ACS grade from Sigma-Aldrich (Sigma-Aldrich Canada Ltd., Oakville, Ontario). Solution volumes were 5mL contained in 10mL polypropylene centrifuge tubes (Simport plastics, Beloeil, Quebec). The experimental solutions were cooled rapidly (between 60-80K/min[23]) by plunging the centrifuge tubes into liquid nitrogen where they were immersed for 30 minutes. Upon retrieval the solutions were visually examined and given a binary score (Table 1). The centrifuge tubes were then immersed in a 37°C water bath until the solution had completely liquefied and given a score on an ordinal scale (Table 2) to evaluate their overall glass stability.

Table 1: Scoring rubric used following plunge of solutions into liquid nitrogen

Vitrification upon	Binary score
Visible Ice	0
No visible Ice	1

Table 2: Scoring rubric used following transfer of solutions into a 37°C water bath

Devitrification upon warming	Ordinal score
No vitrification	0
Complete devitrification	1
Partial devitrification	2
Devitrification at edges	3
No devitrification	4

Statistical methods:

This experiment seeks to gain insight into the relative contributions of individual cryoprotective agents to the vitrifiability and glass stability of multi-CPA solutions so regression analysis was used. Since the vitrifiability and glass stability data are discontinuous the most appropriate statistical tool is logistic regression. In the case of vitrifiability binary logistic regression is most appropriate, while for the evaluation of glass stability ordinal logistic regression is the most appropriate technique. The vitrifiability of solutions was evaluated by creating a binary logistic regression model using binary logistic regression to evaluate vitrification upon cooling (using STATASE by Statacorp, College Station, Texas). Binary logistic regression uses least squares regression to fit coefficients to the independent variables that are being used to predict a binary dependent variable. The model used to predict vitrification upon cooling takes the form:

$$Logit[P] = \alpha + \sum_{i=1}^{k} \beta_i x_i \qquad eq.\ (1)$$

[30]. It should be used in cases such as ours, where the dependent variable has two distinct outcomes with no intermediate outcomes. In *eq.*(1), β_i represents the contribution of a CPA to the vitrifiability of a solution per molar increase in CPA concentration, x_i represents the molar concentration of a CPA and α represents the threshold that separates a statistically vitrifiable solution from an ice forming solution. P is the probability that no ice will occur in the solution and is treated as a binary variable: 0 (ice formation); 1 (vitrification). Logistic regression has the same assumptions as linear regression: that the data are normally distributed and that the errors are independent of each other[30]. Logit is a mathematical transformation of a logarithmic regression formula that allows analysis of the regression coefficients as though they were derived from linear regression formula, though the constraints of logistic regression remain[30] . The glass stability of solutions was evaluated by creating an ordered regression model, using proportional odds ordered logistic regression to evaluate overall glass stability of the solutions. Proportional odds logistic regression uses least squares regression to fit coefficients to the independent variables that are being used to predict an ordinal dependant variable. The model used to predict glass stabilty upon warming takes the form:

$$Logit[P] = \alpha_n + \sum_{i=1}^{l}\left[\beta_i x_i + \sum_{j=1}^{i}\beta_{ij}x_i x_j\right] \qquad eq. (2)$$

used in cases where the dependant variable has more than two states, but is not continuous. In *eq.* (2) α_n represents the threshold that distinguishes which score a solution is likely to receive and *P* is the probability that a solution will not devitrify. When $Logit[P]$ is greater than or equal to one, the equation predicts that the solution will vitrify. If $Logit[P]$ is less than one, the equation predicts that the solution will not vitrify. The β_i represent the contributions of each CPA to the glass stability of a solution per molar increase in CPA concentration The β_{ij} represent interactions between components of the solution. The x_i and $x_i x_j$ represents the molar concentration of a CPA and the product of the molar concentrations of two CPAs respectively. The $\beta_{ij}\ x_i x_j$ term is used to model first-order interactions between CPAs, including interactions of a CPA with itself. The proportional odds ordinal logistic regression model creates several binary regression models, creating binary variables by splitting the ordinal scale at each discrete value. For example, a solution with a score that meets or exceeds the threshold α_3 is predicted to meet or exceed a score of 3. The single ordered model is created by fitting a single set of β coefficients to this collection of binary models.

Models were constructed by adding blocks of variables (individual CPAs, interactions) to the model-in-progress and numerically testing to ensure that there was a statistically significant (set at p=0.95 using F-tests) decrease in the value of the residuals (sum of the squares of the difference between the data and the model). Individual variables of blocks that were added to the model were then tested to ensure that their removal decreased the fit of the model significantly. If this was not the case, the variable was removed per good statistical practice[30].

Results

Table 3 lists the parameters of the binary multiple logistic regression model. The CPA with the greatest incremental contribution to the vitrifiability of a multi-component solution was propylene glycol with a coefficient of 225. Glycerol and dimethyl sulphoxide had the second greatest contribution, both possessing a score of 188. Ethylene glycol had the second smallest contribution to the vitrifiability of a solution with a coefficient of 150 and formamide contributed the least to the vitrifiability of a solution with a coefficient of 75. The parameter α is a threshold; when the sum of the products of the molarities of the CPAs with their coefficients is greater than the absolute value of α, *eq.* (1) will result in a P=1, predicting that the solution will vitrify.

Table 3: Parameters of model predicting vitrification of solutions upon quenching in liquid nitrogen

Parameter	Estimate
α	-1070 ± 0
β_{PG}	225 ± 0
β_{Glyc}	188 ± 0
β_{DMSO}	188 ± 0
β_{EG}	150 ± 0
β_{Form}	75 ± 0

In the multiple CPA solutions tested, a large difference in contribution to the vitrifiability was evident between CPAs, which was evident when the greatest and least effective CPAs were compared. For example, increasing the concentration of propylene glycol in a solution by one molar increased the probability of that solution vitrifying by three times more than does increasing the concentration of formamide in the solution by one molar. This binary model perfectly predicted the vitrification status of all 164 solutions with no variation, resulting in standard errors of 0. Since a single set of these 164 solutions was tested there were no duplicate data points that could have produced variation in the data and hence the model. Nonetheless, it is possible that if multiple sets of the 164 solutions were tested that the model could be identical, with standard errors of zero since the outcome is binary and based upon primarily physical processes.

Table 4 lists the thresholds that the ordinal model predicts a solution must pass in order for the solution to receive a score of 1-4 (Table 2). Standard errors are an estimate of the standard deviation present within the underlying data, the true mean could occur anywhere within the range of the standard error (or within several multiples of the range of the standard error depending on the certainty desired)[30]. Since the standard errors of the estimates were greater than the differences between the thresholds, statistically they cannot be considered to be completely distinct without other tests being completed.

Table 4: Thresholds of ordinal model predicting degree of devitrification

Parameter	Estimate (± SE)
α_1	167.6 ± 29.2
α_2	184.0 ± 31.5
α_3	186.8 ± 31.8
α_4	190.5 ± 32.0

Table 5 lists the contribution of individual CPAs to the glass stability of solutions. The coefficients in this glass stability model followed the same relative CPA order as in the vitrifiability model with the exception that dimethyl sulphoxide had the second smallest contribution to the glass stability of a solution while in the vitrifiability model it had the second highest contribution to the vitrifiability. Note that the coefficients of propylene glycol, glycerol, ethylene glycol and dimethyl sulphoxide all fell within each other's 95% confidence intervals and are not distinguishable at that confidence level. All four were significantly different from the glass stability coefficient of formamide, which contributed the least to the glass stability of solutions that contain it. This suggests that formamide is the least effective of these 5 CPAs at preventing ice formation in multi-component solutions.

Table 5: Linear contributions of CPAs in ordinal model predicting degree of devitrification

Parameter	Estimate (± SE)
β_{PG}	57.1 ± 10.6
β_{Glyc}	45.9 ± 8.6
β_{EG}	39.9 ± 8.0
β_{DMSO}	36.4 ± 6.7
β_{Form}	12.3 ± 2.3

Table 6 lists the coefficients representing the contribution of the interactions between CPAs to the glass stability of solutions. The addition of the interaction terms to the model significantly improved the precision of the model using p=0.05; however, the interactions of dimethyl sulphoxide with formamide and of formamide with itself did not significantly improve the model, resulting in their removal from the model, per standard statistical practices[30].

Table 6: Interactive contributions of CPAs in ordinal model predicting degree of devitrification

Parameter	Estimate (±SE)
β_{PG_Glyc}	-7.0 ± 1.4
β_{PG_EG}	-5.9 ± 1.3
β_{DMSO_PG}	-5.9 ± 1.2
β_{EG_Glyc}	-5.0 ± 1.1
β_{DMSO_Glyc}	-4.3 ± 0.9
β_{PG_PG}	-4.1 ± 0.9
β_{DMSO_EG}	-3.7 ± 0.9
β_{Glyc_Glyc}	-2.9 ± 0.7
β_{EG_EG}	-2.3 ± 0.6
β_{PG_Form}	-2.0 ± 0.5
β_{EG_Form}	-1.7 ± 0.5
β_{DMSO_DMSO}	-1.4 ± 0.4
β_{Glyc_Form}	-1.2 ± 0.4

All interaction terms were negative and a trend was apparent in the interaction terms. When CPAs had larger linear contributions to the glass stability of a solution, they also tended to have more negative coefficients of interaction with other CPAs. Negative interaction coefficients indicate a reduction in the probability of a solution avoiding devitrification as the concentration of a solution increases; this has the effect of offsetting the increase in the probability of a solution avoiding devitrification resulting from the positive linear coefficients of the CPAs as shown in *eq.* (2)[21].

Discussion

The addition of interaction terms to the glass stability model significantly improved the model as measured using F-tests. Previous work investigating the glass stability properties of multi-component CPA solutions, primarily with ternary systems composed of two CPAs in water, assumed that interactions were negligible or did not occur[6; 8; 10; 11; 15]. This may remain true within that experimental system, though this should be verified experimentally; however, since we have found that interactions significantly affected the glass stability of multi-component solutions of CPAs containing physiological levels of salt, the impact of these interactions must not be ignored during the design of multi-component vitrification solutions. These interaction terms were all negative, which indicated that as the concentration of a solution increased the incremental improvement to the glass stability of the solution decreased. This is intuitive if we think of what is happening in solution; increasing the concentration of a solution from 5 Molar to 6 Molar increases the CPA proportion of the solution by a greater amount than will increasing the concentration of the solution from 6 Molar to 7 Molar. CPAs that have large primary glass stabilities also tended to have the most negative interaction terms, suggesting that there was potential benefit in having solutions with several components at low concentrations rather than only a few components at higher concentrations, as interaction terms become multiplicatively more significant to the solution. This is a key insight as many current approaches focus on single or binary solutions for use as vitrifying CPA solutions[18].

The precision of the vitrifiability model was greater than that of the glass stability model. This was to be expected as the process of evaluating the presence or absence of ice at a single temperature point was more precise than the process of evaluating the amount of ice formed over a period of several minutes and over a range of temperatures as a solution was warmed in a water bath. Since the glass stability measurement has a more subjective grading scheme, we expected more observation error and therefore more error in the resulting model. The order of the CPA vitrifiability coefficients largely agreed with published critical cooling rates for the same CPAs in binary solutions[1], with the notable exception of glycerol. Published critical cooling rates for formamide could not be found. Surprisingly, glycerol had the second largest vitrifiability coefficient, while its binary critical cooling rate was the lowest of dimethyl sulphoxide, propylene glycol, ethylene glycol and glycerol. There are possible reasons for this. The published critical cooling rates were collected using binary solutions of a single CPA and water, while our data was collected using solutions of multiple CPAs in DPBS. It is possible that glycerol interacts with other solutes in the solution, resulting in more effective vitrification. A second possible reason for this difference is due to the method of measurement, the data used to derive the published binary critical cooling rates was collected using samples of a few milligrams[1]; whereas this study used samples that were three orders of magnitude larger. Whereas the vitrifiability coefficients are most readily compared to critical cooling rates as they are both benchmarks of the relative ability of solutions to avoid crystallization upon cooling, the glass stability coefficients can be compared to critical warming rates. The published critical warming rates of binary CPA solutions suggest that the relative glass stability of the CPAs (at 45% solute w/w) is, in descending order, propylene glycol (80°C/min), dimethyl sulphoxide (3.3×10^{3} °C/min), ethylene glycol (3.3×10^{5} °C/min) and glycerol (5.6×10^{8} °C/min)[1]. Our glass stability model coefficients followed the same order but dimethyl sulphoxide and glycerol exchanged positions. Nonetheless, care should be taken not to place undue weight on this difference as the 95% confidence intervals of these four coefficients overlapped and so they were not statistically different from one another at this confidence level. This results point to the need to collect a larger data set to narrow the confidence intervals and allow stronger conclusions to be drawn.

Conclusion

We have found that the glass stability of solutions of multiple CPAs do not behave as linear additions of binary solutions but that interactions between CPAs have a significant effect on the glass stability of these solutions. This should be considered when designing vitrification solutions. We have provided values that predict if a solution will vitrify; the rank order of the CPAs' tendency to cause a solution containing multiple CPAs to vitrify agrees with the CPAs' tendency to vitrify in single CPA solutions[1], with the exception of glycerol. From a theoretical standpoint, it is desirable to have multiple solutes in order to avoid crystal formation, since the more distinct solutes are present the less likely it is that molecules of the same substance (whether water or CPA) will be present in sufficient numbers in localized areas to allow crystal growth[18].

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