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Advancing Microwave Technology for Dehydration Processing of Biologics

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Our prior work has shown that microwave processing can be effective as a method for dehydrating cell-based suspensions in preparation for anhydrous storage, yielding homogenous samples with predictable and reproducible drying times. In the current work an optimized microwave-based drying process was developed that expands upon this previous proof-of-concept. Utilization of a commercial microwave (CEM SAM 255, Matthews, NC) enabled continuous drying at variable low power settings. A new turntable was manufactured from Ultra High Molecular Weight Polyethylene (UHMW-PE; Grainger, Lake Forest, IL) to provide for drying of up to 12 samples at a time. The new process enabled rapid and simultaneous drying of multiple samples in containment devices suitable for long-term storage and aseptic rehydration of the sample. To determine sample repeatability and consistency of drying within the microwave cavity, a concentration series of aqueous trehalose solutions were dried for specific intervals and water content assessed using Karl Fischer Titration at the end of each processing period. Samples were dried on Whatman S-14 conjugate release filters (Whatman, Maidestone, UK), a glass fiber membrane used currently in clinical laboratories. The filters were cut to size for use in a 13 mm Swinnex® syringe filter holder (Millipore™, Billerica, MA). Samples of 40 μL volume could be dehydrated to the equilibrium moisture content by continuous processing at 20% with excellent sample-to-sample repeatability. The microwave-assisted procedure enabled high throughput, repeatable drying of multiple samples, in a manner easily adaptable for drying a wide array of biological samples. Depending on the tolerance for sample heating, the drying time can be altered by changing the power level of the microwave unit.

Introduction

Currently, cryopreservation methods (slow-cooling or vitrification), with storage at cryogenic temperatures, remain the standard for preservation of cells and tissues. Although cryopreservation is a proven process, it remains expensive. Specialized cryogenic equipment is required and access to a reliable supply of liquid nitrogen is essential for long-term maintenance of the biological samples. Recent research has shown that anhydrous preservation may be an alternative approach to achieving long-term storage of biological samples.[1,2] This preservation method relies on removing water from solutions that contain glass-forming protectants, such as the disaccharide trehalose. As water is removed from the bulk sample, the remaining sugars and salts become concentrated, and, as long as the solutes do not crystallize, the viscosity increases with progressive water loss until a glassy state is achieved. This dramatically reduces the molecular mobility within the matrix and can thus minimize the degradation of biological material, including proteins, bacteria, viruses, and cells.[3–8] As long as samples are stored below the glass transition temperature of the matrix, biochemical reactions are slowed to the extent that long-term storage becomes possible. In some cases, it is important that the protectants are loaded intracellularly to be most beneficial.[9,10] Some common methods for introduction of trehalose into a cell are fluid-phase endocytosis,[11] micro-injection,[12] and ATP-induced poration.[8]

Anhydrous preservation has the potential to significantly reduce the cost of preserving biological samples, as the maintenance costs would be minimal once the sample was dried to appropriate moisture levels and suitably packaged. The challenge with drying biological material is the need to provide molecular and organelle protection within a suitable glass-forming composition, while also minimizing the chemical and physical stress that the samples are exposed to during dehydration processing. Achieving a homogeneous sample with a uniform moisture content can also be a challenge.[13] Numerous drying approaches have been investigated to achieve a dried specimen suitable for storage in a dry state, including lyophilization,[5,7] drying in controlled humidity desiccators,[14] or the use of high flow nitrogen gas for convective drying.[15,16]

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Recently our group has demonstrated that microwave processing can be used to reproducibly dehydrate samples for preservation in an anhydrous state, yielding samples that are more uniform in water content than those produced by other dry processing methods.[13] The current work expands upon the original proof-of-concept, and details advances in instrumentation and process methodology to enable higher throughput drying with more process control.

Materials and Methods

Microwave instrumentation and drying accessories

A SAM 255 Microwave (CEM, Matthews, NC) was used for all dehydration experiments. This instrument has a built-in infrared sensor (IR), which determines the average temperature inside a designated diameter ($\sim$7 cm) in the cavity. This allows the microwave to be programmed to stop operation if the temperature reaches a designated maximum operational temperature, a useful feature for the processing of thermally sensitive samples. It also has adjustable user specifications, such as power level and fan speed. In the current work the fan was disabled in order to reduce the effect of convective drying. Because of the rotational movement of the turntable (necessary for even microwave exposure) some convective drying component will always be present during microwave processing. It should also be noted that the cavity has ventilation ports, so drying commences in ambient relative humidity conditions. The threshold temperature limit of the instrument was also set high (100°C) to avoid power interruptions during characterization studies, as the built-in IR sensor did not measure a spot size that was suitable for the current study. In order to observe individual sample temperatures, for the purposes of this study a hand-held IR temperature sensor was used instead of the built-in instrument IR sensor.

The container used for drying was a polyethylene syringe filter holder (Millipore, Billerica, MA) of 13 mm diameter (Fig. 1). These containers were selected to enable drying, storage, and rehydration of small volumes of high value biological product within the same container, to minimize handling losses. These devices are reusable, and can be autoclaved between uses. Prior to consideration as a storage container, the assemblies were autoclaved repeatedly (more than $n=7$ cycles), and functionality as a holder was assessed between cycles. These syringe filter holders can be used with any syringe that has a Luer-Lok™tip (Ex. Turumo Medical Corporation, Somerset, NJ). The drying surface chosen was Standard 14 (S-14) borosilicate glass microfiber paper (Whatman, Maidstone, UK) of 17 mm thickness. These filters are designed to absorb liquid through capillary action and release the filtrate upon reintroduction of liquid. The S-14 filters have a conjugate release rate of 75% and water absorption rate of 55 mg/cm^2 (Whatman product literature).

A turntable for the microwave was also fabricated for this application (Fig. 1). It was designed for processing of multiple samples, using both the measurements of the syringe filter holders and the existing design of CEM's turntable as a template. The turntable was made from Ultra High Molecular Weight Polyethylene (UHMW-PE; Grainger, Lake Forest, IL). UHMW-PE was chosen for its low dissipation factor, moderate cost, and ease of machining. This turntable enabled processing of 12 samples at a time.

Solution preparation and sample processing

Sugar solutions were prepared from high purity low endotoxin α-α-Trehalose dihydrate (Ferro Pfanstiehl, Waukegan, IL) using 18.2 MΩ water as the solvent, in concentrations of 1.50, 0.75, 0.50, 0.25, and 0.125 M. Droplets of 40 μL volume were transferred onto Whatman S-14 conjugate release filter paper (Whatman, Maidstone, UK) that had been cut into 13 mm discs using a die cutter. These filters were placed on the bottom half of the syringe filter assembly, which had been positioned in the custom turntable.

With the exception of the power study, all samples were processed with 20% microwave power for timed increments ranging from 0 to 40 minutes, depending on the goal of the experiment. At the 20% power setting the power output was determined to be 108 ± 7 W ($n=5$), measured using a K type thermocouple immersed in a polypropylene beaker containing 500 mL of 18.2 MΩ H_2O. The power at 100% was measured to be 608 ± 21 W ($n=5$). The interior fan and light were disconnected manually. Average sample temperature was measured by a hand-held IR temperature sensor (IRT207 General Tools, NYC, USA; resolution ±2°C) immediately following drying. The water content of samples after microwave processing was measured by Karl Fischer titration by placing the filter papers directly into a Mettler-Toledo V20 Volumetric Karl Fischer Titrator (detection limit 100 ppm). Methanol was used as the solvent and the titrant used was AquaStar CombiTitrant5 (EMD Chemicals, Philadelphia, PA), a one component titrant that enables testing of samples up to 100% water content, while still remaining accurate in the lower limits of water content. Prior to choosing this solvent for all measurements, formamide was added in a 50:50 ratio with methanol and used to quantify water in a 1.5 M

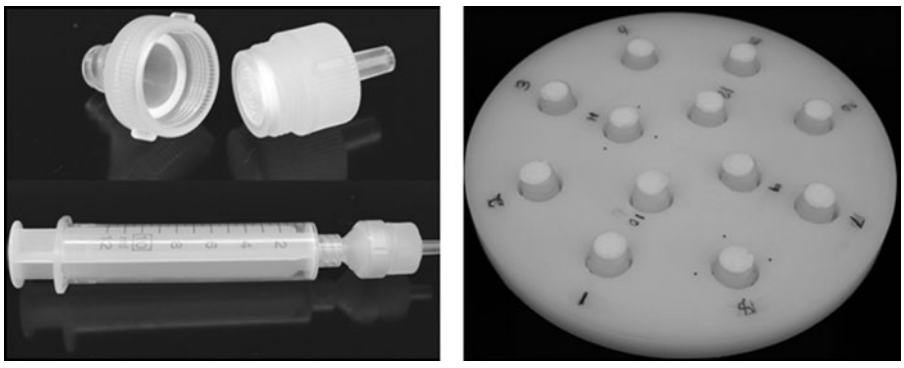

FIG. 1. Millipore's Swinnex 13 mm polypropylene filter housing provides a reusable container that allows for the interchangeability of a variety of drying substrates. Once assembled the filter housing can be attached to a Luer-Lok® syringe for rehydration of dried samples. Filters containing trehalose samples were placed on this filter unit surface in preparation for dehydration processing and then placed in a custom turntable that had been periodically recessed to accommodate the base of the filter unit.

trehalose solution that had been dried to 0.1 g H_20/gdw. Methanol:formamide titration yielded statistically equivalent results ($\alpha = 0.05$) to titration in methanol alone (data not shown). The pre-titration mix time was increased from the factory standard of 30 seconds, to 120 seconds to maximize the time for water release into the methanol before titration. Dry weight for each of the original concentrations was determined by subtraction of the average titrated water content from the average sample mass of a 40 μL aliquot for each of the five trehalose solutions. The average mass was determined by weighing 40 μL droplets of each sugar solution, delivered by Eppendorf pipette, twenty times using a Mettler Toledo AX105 analytical balance. The same volume of each solution was titrated five times, providing a value that could be averaged for water content. The difference between the average hydrated sample mass and the average mass of water titrated yielded the dry mass of trehalose in each given volume of stock solution. The water content was then expressed in terms of grams of water per grams of dry weight (gH_2O/gdw) for all subsequent analyses. While this approach combines the uncertainty in volumetric measurements, weighing, and titration, these are known uncertainties, and can be estimated and managed with an appropriate number of replicates. Furthermore we have determined that this approach yields equivalent results to traditional bake-out measurements for volumes of 40 μL (and higher) with as few as five replicates (data not shown).

The relative humidity was monitored throughout all experiments using a HH314A Humidity Temperature Meter (OMEGA Engineering, Stamford, CT). All experiments were performed at 43.2% RH + / − 5.5.

Sample characteristics and process repeatability

It is recognized that by virtue of the sample size, the extent of absorption of microwave energy is dependent on the quality of microwave reflection and mixing within the cavity. To determine the run-to-run repeatability, within-batch variability, and the effect of sample number on end-point moisture content, samples of 0.500 M trehalose solution of 40 μL volume were dehydrated by processing for 10 and 20 min in sets of 1 (sample placed in position 1 of the turntable) or 12 (entire turntable filled). Experiments were repeated a total of 5 times. Each sample was weighed and then immediately titrated for moisture content after the microwave processing was complete. During each microwaving session, the highest temperature that was measured by the IR sensor was recorded.

Determination of drying rates and end-point moisture content as a function of initial concentration and power level.

Because the hydrated trehalose solutions used in this study are not in moisture equilibrium with the typical ambient environment (22°C, 40% RH) the samples will dehydrate in room air without any other intervention. Drying will proceed until an equilibrium moisture level is reached. Although the samples used in these experiments will ultimately equilibrate to an equilibrium moisture content that is dictated by the environmental RH, the rate at which the sample will equilibrate and the distribution of water within the sample during this process can vary considerably depending on certain environmental factors, including the drying surface and/or matrix and the presence or absence of convective flows. These factors can also influence the formation of glassy 'skins' on the surface of droplets that can impede drying.[17] In the current work, glass fiber papers were used to absorb and distribute the sample, thus minimizing some of the adverse effects associated with droplet drying, and enhancing drying rates overall by increasing the surface area for drying. In order to determine the contribution of microwave energy delivery to the overall drying rate, samples of 0.5 M trehalose in water were processed in 5 min increments until the titrated water content of three consecutive time points were the same. Equivalent samples were also processed by handling the sample identically but without exposing the samples to microwave energy during the drying phase. Samples were then processed as described previously.

To determine the effect of power levels on processing time trehalose samples of 0.5 M concentration were processed for 5 min at 10% incremental power settings from 0% to 100%. Each sample was processed in the microwave individually in position 1 of the turntable, and infrared temperature readings were taken immediately after microwaving using a handheld IR thermometer (13 mm diameter sensing area). Five samples for each power setting were processed.

Results

Sample characteristics and process repeatability

In order to determine the influence of sample position on the drying rate, the end-moisture content measured at each specific position was determined at the end of 10 minutes and 20 minutes of processing. All samples that were microwave processed consisted of 40 μL volumes of 0.5 M aqueous trehalose solution on Whatman S-14 conjugate release filters. As shown in Table 1, after a 10 min processing period a modest but statistically significant difference was measured in end moisture content between the inner ring samples (average of positions 9–12) and the outer ring samples (average of positions 1–8) of the turntable (one-way ANOVA). No statistical difference was observed between individual positions within the outer ring or between individual positions within the inner ring. Assuming a linear decrease in moisture content over this 10 min period of drying, the drying rate would be 2.22 mg H_2O/min for the outer ring and 2.53 mg H_2O/min for the inner ring, a 13% difference. After 20 min of microwave processing there was no difference in the final moisture content for samples dried in the inner and outer ring positions ($p = 0.3443$, two way unpaired T test $\alpha = 0.05$). When a single sample was processed in position 1, the average final moisture content achieved (1.6510 ± .1874 gH_2O/gdw) did not differ from the average value presented in Table 1(1.5520 ± 0.1266 gH_2O/gdw), when the sample was processed within the context of a full turntable of samples ($p = 0.3698$ two tailed unpaired T test $\alpha = 0.05$).

Determination of drying rates and end-point moisture content as a function of initial concentration and power level

The microwave-assisted drying process is a combination of passive forces (diffusion and evaporation driven by chemical potential gradients) and active forces (diffusion and evaporation driven by thermal energy input). In Fig. 2, the

Table 1. The Effect of Turntable Position on the Moisture Content Achieved in a 40μL Droplet of 0.500 M Trehalose After 10 or 20 Mins of Processing Time*

Turntable Position	*Moisture Content g H_2O/g dry weight (± Replicate Std. Dev)*
Processing Time: 10 min	
Outer Ring	
1	1.65 (±0.14)
2	1.72 (±0.25)
3	1.51 (±0.24)
4	1.60 (±0.36)
5	1.40 (±0.14)
6	1.38 (±0.18)
7	1.58 (±0.16)
8	1.41 (±0.27)
Inner Ring	
9	1.05 (±0.29)
10	1.14 (±0.07)
11	1.05 (±0.17)
12	1.26 (±0.31)
	Mean (± Sample Std. Dev)
Processing Time: 10 min	
Outer Ring (n = 8)	1.53 (±0.13)**
Inner Ring (n = 4)	1.13 (±0.10)**
Processing time: 20 min	
Outer Ring (n = 8)	0.15 (±0.01)
Inner Ring (n = 4)	0.16 (±0.02)

*Samples were processed at 41.1 + / − 5.6% RH.
**Differences that are statistically significant (2-way unpaired t-test, $p < 0.0001$).

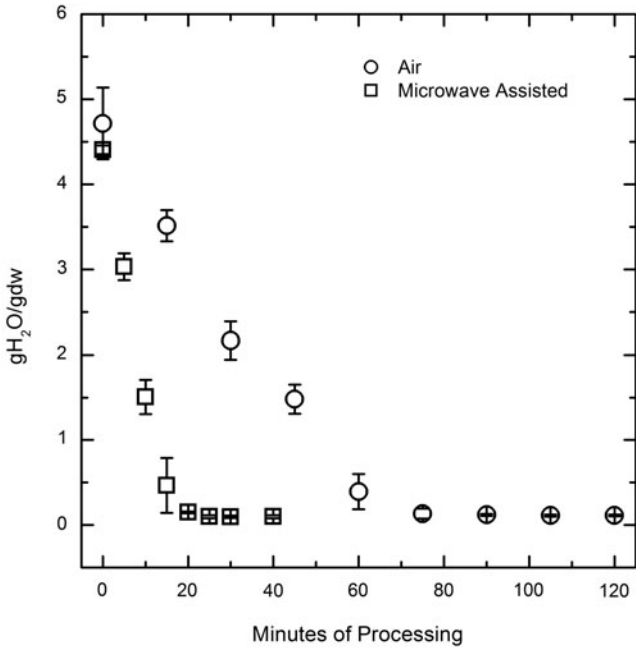

FIG. 2. Moisture content as a function of time for passive (air) and active (microwave-assisted) drying of 40 μL droplets of 0.50 M trehalose solution. Samples dried in the microwave were titrated at the end of each microwave processing period. In the case of the air-dried samples a single filter was selected and titrated every 15 min. All passively dried samples were processed at 42.9 ± 1.3% RH. Microwave-assisted samples were dried at 44.1 ± 3.4% RH. The data points for both processing techniques consisted of five samples each ($n = 5$) with the error bars representing the standard deviation in the calculated moisture contents.

relative contribution of microwave energy delivery towards the overall drying rate can be observed. As shown in Fig. 2, trehalose samples that were dried in the microwave cavity, without microwave power, required approximately 75 min to achieve the equilibrium moisture level of 0.1167 gH_2O/gdw at a RH of 42.9 + / − 1.3%. Microwave-assisted processing at 20% power lowered this time to approximately 25 min under the same conditions (44.1 + / − 3.4%RH). The temperature of individual samples was monitored after different durations of drying in parallel experiments conducted under similar but not identical humidity conditions (49.66 + / − 0.01%RH). At this RH trehalose samples of 0.500 M concentration, starting at an ambient temperature of 22.3°C, experienced a temperature increase of 7°C through the first 15 min of microwave processing. After 15 min the temperature decreased to 28.7 °C where it stayed constant from 25 to 40 min of processing. It should be noted that because measurements were taken with a hand-held IR sensor, there are several seconds of delay between the end of processing and the acquisition of sample temperature because of the need to position the sample and sensor appropriately. Because of this delay, recorded temperatures are expected to be several degrees cooler than the maximum temperature reached at the end of processing. These measurements should thus be considered as approximate indicators of temperature escalations.

The influence of the power setting on the drying time was also investigated. Trehalose samples of concentration 0.50 M and 40 uL volume were processed for 5 min at a range of power settings. The drying rate, as inferred from the end-moisture content after 5 minutes, increased with power up to the 50% power level (nominally 300 W). Beyond this power level no further increase in drying rate was achieved. As shown in Fig. 3, the maximum temperature recorded was 35 °C, which was observed after 5 minutes of processing at 80% power. These recordings were consistent with thermocouple readings acquired on sacrificial samples that were equivalently processed. Differences between sensors were less than 3°C and within the error range of each sensor. The IR sensor measurements were systematically higher than the thermocouple measurements. However, perfect agreement between sensors was not expected due to differences in the localization of the temperature measurement.

The initial moisture level in the samples will influence the overall drying time. The gradient in the chemical potential of water provides the driving force for evaporation, but the rate at which water exits will depend also on diffusion through the sample and sample characteristics at and near the liquid-air interface. Depending on the balance of these influences, macroscopic changes can arise during processing that accelerate or retard further drying. These phenomena can also be affected by the initial concentration. Trehalose samples with concentrations between 0.25 M and 1.50 M were processed in 5 min increments at 20% power

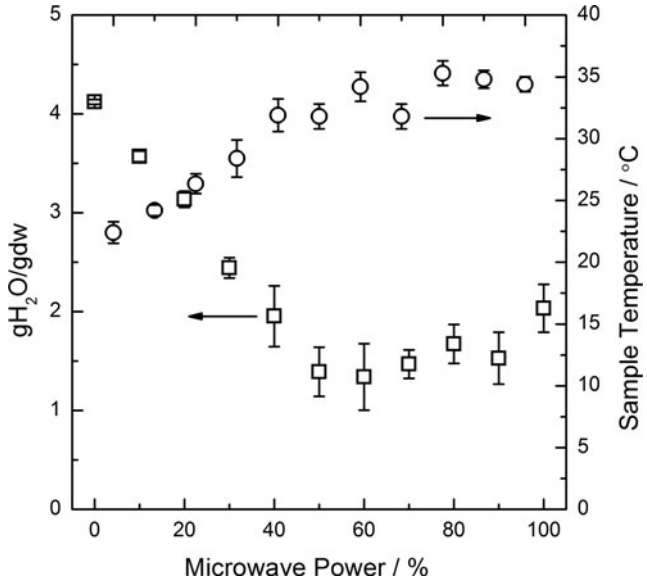

FIG. 3. Effect of microwave power setting on the final moisture content of dried 0.50 M aqueous trehalose solutions and the surface temperature recorded directly after processing. All samples were processed for 5 min in a 44.0 ± 2.9% RH environment. All data points consisted of five replicates ($n = 5$) with error bars representing the standard deviation. A one-way ANOVA performed on the samples in 50% to 90% power levels sets showed no significant variance between these values (f = 1.38, f critical = 2.86 $p = 0.27$).

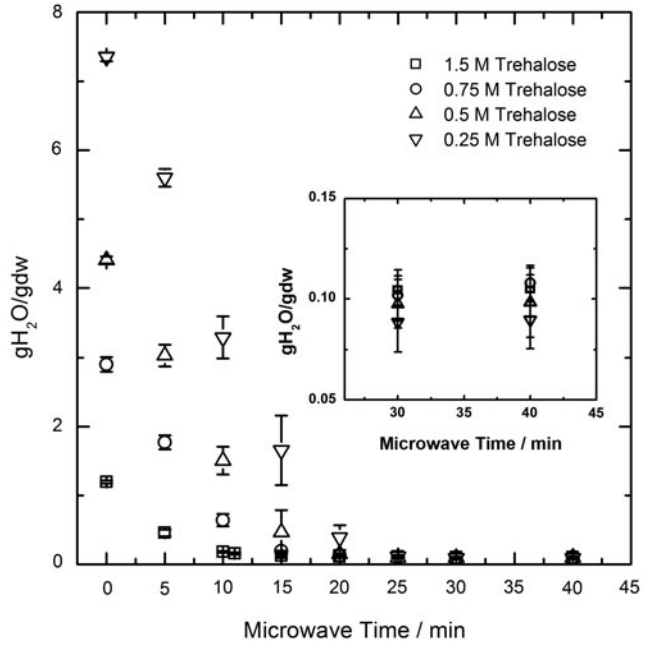

FIG. 4. Effect of trehalose concentration on microwave drying time. Samples consisted of 40 μL volumes of 1.50 M, 0.75 M, 0.50 M, and 0.25 M concentration. All samples were processed at 44.7 ± 3.8 RH. The inset graph is a zoom enhanced region of the data points from 30 to 40 minutes. Error bars represent the standard deviation of replicates ($n = 5$).

until mass loss was insignificant. As shown in Fig. 4, drying durations followed expected trends; the time required to achieve a consistent moisture content decreased as the concentration increased.

It should be noted that the average moisture content converged to 0.1 g H2O/gdw for all trehalose concentrations after 40 min of processing time in the microwave (Fig. 4 inset). This value is consistent with the equilibrium moisture content determined previously for binary trehalose water solutions from 10% to 90 % relative humidity levels.[18] Binary solutions of trehalose and water are characterized as having a type III sorption isotherm.

Discussion

In previous work, we demonstrated the utility of microwave-enhanced drying as an approach for dehydration of biological suspensions intended for dry state storage.[13] This approach yielded a more uniform dried product, and the drying rates achieved were very reproducible. However, processing was limited to a single sample at a time and the drying approach required manual stop and start operation to control temperature. In the current work, we have utilized a microwave processor with variable power control and have scaled the process to enable batch processing of 12 samples. Also, instead of drying samples in droplets, aliquots were absorbed onto glass fiber filter paper, thus maximizing the surface area for evaporation and minimizing surface tension effects. The samples were then processed using continuous low power wherein the heating effects of microwave delivery were simultaneously modulated by the cooling effects of evaporative water loss, resulting in only modest temperature rises in the sample during processing. By contrast, in the case of static air-drying the sample would be dominated by the effects of evaporative cooling, and this would serve to lower the temperature and ultimately slow down the rate of drying. In the current approach the net effect of microwave heating and evaporative cooling maintains the sample temperature, yielding improved overall drying rates. While this thermal titration effect could theoretically also be achieved by contact heating of the sample, microwave energy input has been shown to effectively interrupt gradient-driven mass transfer processes that lead to sample inhomogeneity, likely due to the volumetric nature of this energy delivery.[13] It is not clear whether or not contact heating would yield this same beneficial overall effect.

When samples were processed in batches of 12, a statistically significant difference was found between samples positioned in the inner ring as opposed to the outer ring for intermediate drying levels (10 minutes of microwave processing at 20% power), but the difference was moderate (2.22 and 2.53 mg H_2O/min for the outer ring and inner ring, respectively). This difference may be of no biological consequence, as there have been very few systematic studies of the influence of drying rates on cell survival during dehydration. Investigations of the effects of cumulative osmotic stress on biologics would suggest that faster drying rates would be more ideal from the standpoint of minimizing the cumulative osmotic stress that biologics would undergo during drying.[19] Also, rates should obviously be fast enough to minimize the probability of sugar crystallization (generally an undesirable outcome for preservation purposes), but also slow enough to permit adequate diffusion of water from the

interior of samples and the transport of water across membranes. The existence of an optimal drying rate is purely speculative at this stage, but, if it exists, it would probably be highly dependent on the composition and cell type. Although samples positioned in the inner ring dried slightly faster than those in the outer ring during the initial 10 min of processing, no statistical difference was found between samples in the individual positions that comprised each ring respectively, suggesting that the difference may be due to the distribution of microwaves within the cavity. After processing for 20 min, samples in all positions achieved the same moisture content. It was also determined that there was no statistical difference between microwave processing one sample in the turntable compared to processing a full turntable. Processing one sample at a time or 12 samples simultaneously produced the same dryness level after 10 min, indicating that the drying process in a given sample was not affected by the local evaporative water losses in adjacent samples under the conditions evaluated.

Different sugar concentrations were dehydrated to investigate the possibility of variability in drying rates in solutions of different viscosity. The drying curves followed expected trends, with the time required to achieve a given end-moisture content increasing as the initial water content increased. These studies also demonstrated the repeatability of the microwave-assisted process with standard deviations of less than 0.012 gH_2O/gdw observed on repeat samples at all concentrations tested after 40 minutes of microwave processing. Experiments performed at increasing power levels indicated that faster drying rates could be achieved by increasing the power level, but with some increase in sample temperature. The microwave-assisted dehydration process yields a dried product in a fraction of the time that would be achieved with passive drying in the same environment. Desirable moisture contents suitable for ambient storage can be achieved with continuous microwave-assisted processing in as little as 15 min for 1.50 M trehalose in water and in 25 min for more dilute concentrations in the range of 0.250 M to 0.125 M. With the new turntable, samples could be processed in batches of 12, enabling high throughput reproducible processing compared to the previous methodology.

For all samples processed with 20% power, the temperature measurements did not deviate appreciably from ambient temperature throughout 40 min of drying. Although microwave energy was absorbed in the samples, resulting in a temperature rise due to water dipole rotation, evaporative cooling also served to modulate this increase in temperature. As the power level was increased, samples warmed faster. Depending on the nature of the preserved biologic, these thermal effects will need to be considered when increasing the drying rate with power level settings. It should be noted that the temperatures recorded in these experiments were average surface temperatures and were intended only to demonstrate that heating effects were moderate under the studied conditions. Resolution of the thermal environment within 40 μL droplets was considered beyond the scope of the current study. It is recognized that the potential for small scale temperature non-uniformities exist and this should be considered in instances where moderate deviations in temperature can be significant for the desired application. Although the microwave-assisted drying approach was initially developed to process cells into a dry state for anhydrous storage, the methodology can easily be adapted to the preservation of small volumes of any valuable biological product, including biomedically-relevant viruses.[20]

Conclusions

The use of microwave processing of mammalian cells is a new approach for drying biological samples into a dry state for extended storage. In the current work we have presented advances in microwave-assisted drying to support its application for a broad range of biological dry storage applications in which small volume uniform products with predictable moisture contents are important.

Author Disclosure Statement

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The authors have no conflicts of interest or financial ties to disclose.

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