Biochimica et Biophysica Acta 1778 (2008) 2283–2290

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Roles of membrane structure and phase transition on the hyperosmotic stress survival of *Geobacter sulfurreducens*

Vishard Ragoonanan [a], Jason Malsam [a], Daniel R. Bond [b,c], Alptekin Aksan [a,*]

[a] *Biostabilization Laboratory, Mechanical Engineering Department, University of Minnesota, Minneapolis, MN, USA*
[b] *Department of Microbiology, University of Minnesota, St. Paul, MN, USA*
[c] *Biotechnology Institute, University of Minnesota, St. Paul, MN, USA*

ARTICLE INFO

Article history:
Received 25 February 2008
Received in revised form 23 May 2008
Accepted 9 June 2008
Available online 14 June 2008

Keywords:
Osmotic stress
FTIR
Phase transition
Lyotropic
Dehydration

ABSTRACT

Geobacter sulfurreducens is a δ-proteobacterium bacteria that has biotechnological applications in bioremediation and as biofuel cells. Development of these applications requires stabilization and preservation of the bacteria in thin porous coatings on electrode surfaces and in flow-through bioreactors. During the manufacturing of these coatings the bacteria are exposed to hyperosmotic stresses due to dehydration and the presence of carbohydrates in the medium. In this study we focused on quantifying the response of *G. sulfurreducens* to hyperosmotic shock and slow dehydration to understand the hyperosmotic damage mechanisms and to develop the methodology to maximize the survival of the bacteria. We employed FTIR spectroscopy to determine the changes in the structure and the phase transition behavior of the cell membrane. Hyperosmotic shock resulted in greatly decreased membrane lipid order in the gel phase and a less cooperative membrane phase transition. On the other hand, slow dehydration resulted in increased membrane phase transition temperature, less cooperative membrane phase transition and a small decrease in the gel phase lipid order. Both hyperosmotic shock and slow dehydration were accompanied by a decrease in viability. However, we identified that in each case the membrane damage mechanism was different. We have also shown that the post-rehydration viability could be maximized if the lyotropic phase change of the cell membrane was eliminated during dehydration. On the other hand, lyotropic phase change during re-hydration did not affect the viability of *G. sulfurreducens*. This study conclusively shows that the cell membrane is the primary site of injury during hyperosmotic stress, and by detailed analysis of the membrane structure as well as its thermodynamic transitions it is indeed possible to develop methods in a rational fashion to maximize the survival of the bacteria during hyperosmotic stress.

1. Introduction

Geobacter sulfurreducens, *Rhodoferax ferrireducens*, and *Shewanella oneidensis* are capable of transferring electrons across the cell membrane [1–5]. In particular, *G. sulfurreducens* can oxidize organic compounds and transfer electrons directly to metal surfaces even in the absence of soluble mediators [1]. This makes *G. sulfurreducens* a valuable bioremediative agent for oxidation of contaminants and reduction of toxic metals [6–8]. *G. sulfurreducens* can also be used to generate electricity from waste organic matter in remote biosensors or as biofuel cells [1,9,10]. Such biotechnological applications require successful encapsulation and stabilization of the catalytically reactive bacteria in thin protective coatings on electrode surfaces [11] and in flow-through bioreactors. It is also of utmost importance to develop effective preservation methods for the storage of the coatings that contain the encapsulated bacteria.

Latex coatings can be used to form hyperporous biocatalytic matrices to encapsulate reactive bacteria [12,13]. The hyperporous matrix enables rapid diffusion of chemicals while mechanically protecting and isolating the bacteria from the solution environment [7]. The reaction rates in biocatalytic coatings are dependent on the porosity of the matrix [14]. To adjust the porosity, carbohydrates (such as sucrose, sorbitol or trehalose) are incorporated in the latex formulation during the encapsulation process [14]. The pore-forming ability of the carbohydrates is attributed to their high water affinity, which generates a "water buffer" in the matrix [7]. An added benefit of the carbohydrates is their protective capability against dehydration. It is known that carbohydrates can protect liposomes [15], isolated biological membranes [16], isolated organelles [17], bacteria [18,19] and mammalian cells [20,21] against dehydration stress. It is generally accepted that carbohydrates stabilize the biological structures and protect the organisms during dehydration by replacing the water surrounding the biomolecules and the membranes [22–24] and by dampening the molecular motions [25].

Inevitably, osmotic, mechanical, and thermal stresses are formed during the encapsulation process due to polymerization of the coating

* Corresponding author.
E-mail address: aaksan@me.umn.edu (A. Aksan).

0005-2736/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2008.06.006

material, and the presence of carbohydrates in the surrounding medium. These stresses reduce the viability and the reactivity of the encapsulated bacteria. Since the cell membrane is the primary site for hyperosmotic injury [26,27] the structural changes it undergoes and its integrity are of primary interest. It is proposed that the de-/rehydration kinetics as well as the environmental temperature determine the structure and the integrity of the cell membrane and therefore directly affect the survival of the bacteria during exposure to hyperosmotic stress [28–30].

Cell membrane lipids are highly hydrated. Therefore, individual lipid mobility and collective lipid dynamics (i.e., the packing structure and organization) depend on the activity of the surrounding water [31]. When the cell membrane is fully hydrated it is dominantly in the liquid crystalline (l.c.) phase. However, it transitions into the gel phase with reduction in temperature (thermotropic transition) or water activity (lyotropic transition). The liquid crystalline state is characterized by increased lipid head group spacing, increased disorder in the lipid acyl chains, and a small bilayer thickness [32,33]. In the gel phase the lipid head groups are very tightly packed, the lipid acyl chains become straighter and ordered, and the bilayer thickness increases [32,33]. The direct influence of the osmotic stress on the cell membrane is documented in bacteria with the observation of endocytotic vesiculation and membrane fluidity change in osmotically stressed *Escherichia coli* [28,29], increased membrane fluidity in osmotically stressed *Bradyrhizobium japonicum* [30], and increased membrane phase transition temperature in air-dried *Lactobacillus bulgaricus* [19]. Membrane phase transition is believed to initiate lipid phase separation [34], and membrane fusion [34], directly affecting the viability of the dehydrated organism. Also due to co-existence of the different phases during a phase transition, membrane permeability increases and cells become leaky [35]. Leaking of the cellular contents during rehydration has been identified as the major reason for reduction in viability [34].

The structure of the membrane, and its lyotropic and thermotropic phase transitions can be monitored using fluorescence microscopy [36], Differential Scanning Calorimetry [37], and Fourier Transform Infrared (FTIR) spectroscopy [38]. FTIR spectroscopy has been successfully used to measure the lipid phase transition temperatures of liposomes [15], pollen [39], yeast [40] sperm [41] and bacteria [19,27]. IR spectra, in addition to supplying information on the membrane state and transition kinetics, also contains information on the secondary structures of the cellular proteins [42,43]. The ability to nondestructively and simultaneously quantify the changes in different cellular structures makes FTIR the method of choice in our experiments.

Lyophilization and cryopreservation are the preferred methods for the preservation of bacteria [44]. However, lyophilization and cryopreservation require processing at cryogenic temperatures. To reduce the processing and storage costs, alternative preservation methods such as air, foam, spray and fluidized bed drying have been proposed (see Morgan et al., 2006 [44] for a review of the last three methods). Of all the drying methods listed, air drying is the most feasible and advantageous one for the preservation of the latex-encapsulated *G. sulfurreducens*. The costs associated with cryopreservation can be eliminated if *G. sulfurreducens* can be successfully encapsulated, preserved in a dehydrated state, and stored at room temperature. Therefore, development of methods to increase the resistance of the cells to dehydration stress is crucial not only for encapsulation in coatings but also from preservation point of view.

In this study, we explored the mechanisms of hyperosmotic stress-induced viability loss in *G. sulfurreducens* in order to maximize its survival during encapsulation and preservation in a desiccated state. We employed FTIR spectroscopy to investigate the structure and the thermodynamic phase transitions of the cell membrane. We have determined the changes in the membrane phase transition temperature (T_m), membrane phase transition cooperativity (ξ), and lipid order as a function of the magnitude and the rate of the extracellular osmotic stress, and dehydration/re-hydration temperatures. The values chosen for these parameters mimicked the conditions encountered during encapsulation in latex coatings [7].

2. Materials and methods

2.1. Bacterial culture

G. sulfurreducens strain PCA (ATCC #51573) was sub-cultured in our laboratory at 30 °C using a vitamin-free anaerobic medium containing (per 1 l of solution): 0.38 g KCl, 0.2 g NH_4Cl, 0.069 g $NaH_2PO_4.H_2O$, 0.04 g $CaCl_2.2H_2O$, 0.2 g $MgSO_4.7H_2O$, and 10 ml of a mineral mix (containing per 1 l of solution: 0.1 g $MnCl_2.4H_2O$, 0.3 g $FeSO_4.7H_2O$, 0.17 g $CoCl_2.6H_2O$, 0.1 g $ZnCl_2$, 0.04 g $CuSO_4.5H_2O$, 0.005 g $AlK(SO4)_2.12H_2O$, 0.005 g H_3BO_3, 0.09 g Na_2MoO_4, 0.12 g $NiCl_2$, 0.02 g $NaWO_4.2H_2O$, and 0.10 g Na_2SeO_4). Acetate (as electron donor) was added to the solution at a concentration of 20 mM. The pH of the culture media was adjusted to 6.8, 2 g/l $NaHCO_3$ was added, and the media was flushed with oxygen-free N_2/CO_2 (80/20 v/v) prior to sealing with butyl rubber stoppers and autoclaving. Within 3 h of reaching maximum optical density ($OD^{600} > 0.6$), 10 ml aliquots of the cell suspension were centrifuged for 5 min at 5000 *g*. The cells were then pelleted and re-suspended in either 20 µl of fresh culture medium, or culture medium containing sucrose (see below). Solid media (used in measuring viability by colony forming units) was prepared by adding 1.5% agar (Difco Corp., Lawrence, KS) to the culture media, and pouring into petri plates inside an anaerobic chamber.

2.2. Application of hyperosmotic shock and slow dehydration

In order to investigate the effects of hyperosmotic shock on *G. sulfurreducens* viability, pelleted bacteria were re-suspended in growth medium that contained 0% (as the control group), 6.75, 13.5, or 27% w/w sucrose. In order to investigate the effects of slow dehydration, 20 µl solutions containing *G. sulfurreducens* were air-dried up to 45 min in growth medium that contained 0, 6.75 or 13.5% w/w sucrose. The drying temperature (T_d) was 30 °C and the environmental relative humidity (RH) was kept constant at 65%. Dried samples were re-hydrated at a re-hydration temperature (T_r) of 30 or 40 °C. In order to investigate the effects of drying temperatures, 20 µl solutions containing *G. sulfurreducens* were dried in growth medium that contained 0% (as the control group) or 6.75% w/w sucrose at T_d=5 °C and RH=11%, and were re-hydrated either at T_r=5 °C or 30 °C. The environmental RH was adjusted to 11% for the T_d=5 °C case so that the partial pressure of water in the environment was the same as in T_d=30 °C, 65% RH case. This ensured that the drying kinetics and the water content at the final state were identical in both drying temperatures. Drying times at each temperature were also varied (0, 15, 30, 45 or 60 min) to achieve samples of different final state water content and activity.

2.3. G. sulfurreducens viability measurements

G. sulfurreducens viability experiments were performed in an anaerobic box (flushed with H_2:CO_2:N_2 5:20:75) after the dried samples were re-hydrated with growth medium at different temperatures (T_r=5 °C, 30 °C or 40 °C) by adding 1 ml of growth medium. Re-hydrated bacteria were then incubated for 30 min at either 5 °C, 30 °C or 40 °C. A fluorescence-based viability assay (Baclight L7012; Molecular Probes, Portland, OR) was used to determine cell viability. The viability assay utilized the change in the permeability of *G. sulfurreducens* membrane to specific dyes as a measure of viability. The assay consisted of two dyes: Propidium Iodide and SYTO 9, which were mixed at a 1:1 molar ratio. Fluorescence from the live *G. sulfurreducens* population was measured at an excitation wavelength

Table 1
Effect of Hyperosmotic Shock on *G. sulfurreducens* Viability (n: number of samples in each group)

Extracellular Sucrose Concentration [% w/w]	Extracellular Osmolality [mOsm]	Viability [%]	Significance
0 (n=4)	300	100±0	-
6.75 (n=4)	500	95±8.9	0.163
13.5 (n=4)	700	63±4.7	0.0003
27 (n=3)	1100	41±6.5	0.002

of 430 nm and an emission wavelength of 530 nm using a fluorescent plate reader (Spectra Max, M2, Molecular Devices Company, Sunnyvale, CA). Fluorescence from the dead/permeable *G. sulfurreducens* population was measured at an excitation wavelength of 430 nm and an emission wavelength of 630 nm. Measurements were calibrated during each experiment by using hydrated control cells (100% live) and cells exposed to 70% ethanol (100% dead). *T*-test (two-sample test, assuming unequal variances) was applied to determine the statistical significance (*P*) of the experimental results. Comparison of the viability determined by the fluorescent live/dead assay to that measured by regular plate counts (based on cell growth on agar plates) demonstrated a linear, one-to-one correlation between the two methods (data not shown) showing the validity of the fluorescence viability assay.

2.4. Determination of the phase, lipid order and thermodynamic transitions of the membrane

Temperature ramp Fourier Transform Infrared Spectroscopy (FTIR) measurements were performed on osmotically shocked or slowly dehydrated *G. sulfurreducens* to monitor the changes in the structure and the phase transition behavior of the cell membrane. 20 µl of the experimental solutions containing *G. sulfurreducens* were deposited on CaF_2 windows and dried under specified RH and temperature conditions for different lengths of time matching the conditions in the viability experiments. After drying, the samples were sandwiched between two CaF_2 windows and placed in a controlled temperature cell. FTIR spectra were collected in the 930–8000 cm^{-1} range using a Thermo-Nicollet 6700 spectrometer, equipped with a DTGS detector (Thermo-Nicolet, Madison, WI, USA). The temperature of the sample was recorded using a thermocouple that was located in the controlled temperature cell. FTIR spectra was recorded at 1 min increments while the sample was initially cooled from ambient temperature (22 °C) down to 0 °C, and then heated at a constant rate of 2 °C/min to 100 °C. At each temperature data point an IR spectrum was obtained by averaging 32 scans.

G. sulfurreducens membrane lipid order which refers to the order/straightness of the lipid acyl chains, was quantified by measuring the location of the νCH_2 symmetric stretching band maximum [38], which was located approximately at 2850 cm^{-1} in the control sample. Higher νCH_2 wavenumber/frequency indicates decreased lipid acyl chain order [45]. The change in the temperature dependence of the νCH_2 peak wavenumber was used to determine the membrane phase transition temperature (T_m). Cooperativity of the membrane phase transition (ξ) was determined by measuring the slope of the νCH_2 wavenumber change at T_m. A more cooperative phase transition was indicated by a larger slope at the membrane phase transition temperature.

2.5. Water content analysis

Water contents of the dehydrated samples were determined gravimetrically and spectroscopically. Gravimetric analysis was performed by measuring the weight of the samples before and immediately after drying and also after baking for 2 h at 110 °C (to remove all the water in the sample). The average water contents reported were the averages of three independent gravimetric experiments. In addition, spectroscopic analysis was used to obtain a measure of the ratio of water per cell by ratio of the combination water band area (1900–2300 cm^{-1}) to the peak intensity of νCH_2 peak. The peak intensity of the νCH_2 peak was calculated from the second derivative peak intensity [46].

3. Results

3.1. Effect of hyperosmotic shock on G. sulfurreducens viability and membrane phase transition behavior

Experiments were first conducted to evaluate the effects of rapid hyperosmotic shock on the viability of *G. sulfurreducens*. Cells were exposed to growth medium solutions containing sucrose concentrations up to 27%. Based on live/dead fluorescence analysis, viability of *G. sulfurreducens* was not significantly affected by the presence of up to 6.75% extracellular sucrose (Viability=95±9%), showing that *G. sulfurreducens* could tolerate mild hyperosmotic shock. However *G. sulfurreducens* viability decreased significantly with further increase in the extracellular sucrose concentration (Table 1).

FTIR spectroscopy was used to quantify the membrane response to hyperosmotic shock. Control cells suspended in growth medium had cooperative membrane phase transition (ξ=0.058±0.001 cm^{-1}/°C) at T_m=23±1 °C (Fig. 1). As *G. sulfurreducens* do not have membrane bound organelles, the temperature-induced transition in the CH_2 peak wavenumber is associated exclusively with the l.c. to gel phase transition of the cellular membrane. A membrane phase transition located at 23 °C for the unstressed cells indicated that, as expected, the cell membrane was almost completely in the l.c. phase at the growth temperature (30 °C).

There was no significant difference in the T_m values for *G. sulfurreducens* suspended in growth medium containing 0, 6.75 or 13.5% sucrose (Fig. 1). However, membrane lipids were less ordered in the l.c. phase for the control cells (0% sucrose) compared to the cells suspended in 13.5 and 27% sucrose solutions (as indicated by the high νCH_2 wavenumbers [47]). In the gel phase however, there was no significant difference in terms of lipid order between the cells suspended in 0, 6.75 and 13.5% sucrose solutions. There was no significant difference in the ξ values for *G. sulfurreducens* suspended in growth medium containing 0 and 6.75% sucrose. Exposure to 13.5% extracellular sucrose decreased the cooperativity of the membrane phase transition (ξ=0.053±0.001 cm^{-1}/°C) compared to the control cells (ξ=0.058±0.001 cm^{-1}/°C). In the presence of 27% sucrose,

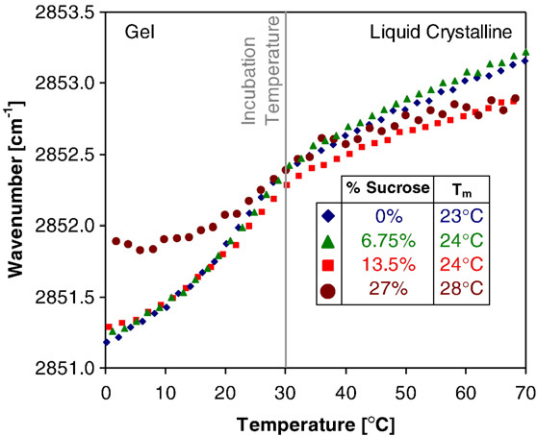

Fig. 1. Change in νCH_2 band maxima with temperature for *G. sulfurreducens* in growth medium (♦),growth medium+6.75% w/w sucrose (▲), growth medium+13.5% w/w sucrose (■), and growth medium+27% w/w sucrose (●) (Representative data set).

membrane phase behavior was drastically different with a higher membrane phase transition temperature (T_m = 28 °C ± 1 °C), significantly reduced lipid order in the gel phase and reduced cooperativity (ξ = 0.033 ± 0.001 cm^{-1}/°C). These results showed the changes in the properties of *G. sulfurreducens* membranes during hyperosmotic shock and revealed that extracellular sucrose directly affected the membrane structure and its thermodynamic phase transitions.

3.2. Effect of dehydration on G. sulfurreducens viability and membrane phase transition behavior

In order to determine the effects of gradually increased osmotic stress, and to test for possible protection capability of sucrose during dehydration, *G. sulfurreducens* suspended in growth medium (0% sucrose) and growth medium containing 13.5% sucrose were dried at T_d = 30 °C, and 65% RH for different time periods up to 1 h. Viability of the dried bacteria was determined after re-hydration at T_r = 30 °C. In both experimental groups viability decreased with increased drying time (Fig. 2A). At all timepoints the viability of the growth medium group was higher even though the difference between the two groups started to diminish after 45 min of drying.

Gravimetric analysis of bacteria revealed that growth medium samples dried for 1 h had 0.55 ± 0.14 $g_{water}/g_{drymatter}$. It is reported that 0.3 $g_{water}/g_{drymatter}$ is the critical water content below which a significant reduction in viability is observed for *E. coli* and *Serratia marcescens* [22,48]. The continuous decrease in viability at all drying times beyond 15 min demonstrate that *G. sulfurreducens* was sensitive to osmotic stress via slow dehydration.

Temperature ramp FTIR analysis was also performed on the *G. sulfurreducens* samples dried (65% RH, T_d = 30 °C) in growth medium and growth medium + 13.5% sucrose solutions. For both treatments,

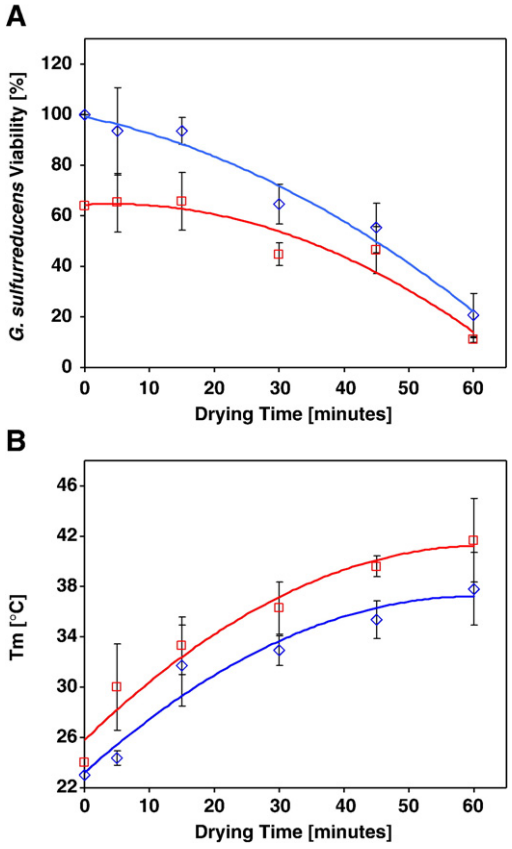

Fig. 2. Change in *G. sulfurreducens* (A) viability, and (B) T_m with drying time for samples dried at 30 °C and 65% RH for up to 60 min in growth medium (◊) and growth medium containing 13.5% w/w sucrose (□) (n = 3 in all data points. Lines are guides for the eye).

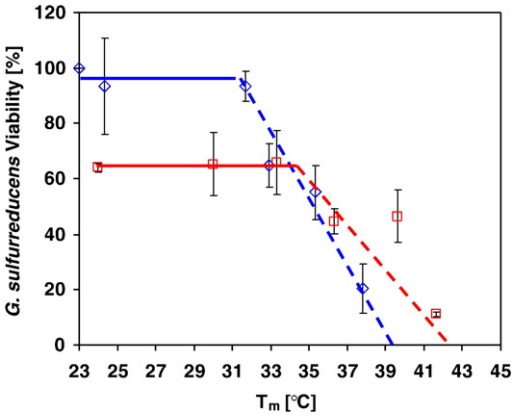

Fig. 3. Change in viability with T_m for *G. sulfurreducens* samples dried at 30 °C and 65% RH for up to 60 min in growth medium (◊) and growth medium + 13.5% w/w sucrose (□) (lines are drawn as guides for eye; error bars for T_m are not shown for clarity; range of error: 1 °C–3.5 °C).

drying increased membrane T_m values (Fig. 2B). At all drying times, average T_m values for *G. sulfurreducens* dried in growth medium + 13.5% sucrose solution were higher than the cells dried in the absence of sucrose. After 45 min of drying, T_m values reached equilibrium, possibly due to equilibration of the solution water activity with the surroundings. The higher T_m values for cells dried in the presence of sucrose revealed that extracellular sucrose posed additional osmotic stress on the membrane, and did not have membrane protection capability when it was present extracellularly.

Fig. 3 shows the post re-hydration viability of *G. sulfurreducens* as a function of T_m. When *G. sulfurreducens* was partially dried in growth medium, and the membrane phase transition temperature rose as high as T_m = 32 °C, viability was not affected (i.e., *G. sulfurreducens* could tolerate mild hyperosmotic stress without loss of viability). Above T_m = 32 °C, there was a negative linear correlation between *G. sulfurreducens* viability and the dehydrated state membrane phase transition temperature.

Similar behavior was also observed for *G. sulfurreducens* dried in 13.5% sucrose solution. The absolute viability of these cells differed at T_m = 23 °C due to the initial osmotic shock. For T_m values greater than 32 °C, cooperativity values for the membrane phase transition were not significantly different for all the sampled dried in the growth medium (ξ = 0.053 ± 0.006 cm^{-1}/°C) and also for those dried in growth medium + 13.5% sucrose (ξ = 0.049 ± 0.006 cm^{-1}/°C). This graph revealed that while the initial hyperosmotic shock (rapid exposure to 13.5% sucrose) altered the membrane integrity (indicated by the lower initial viability), remaining cells retained the ability to withstand slow dehydration, so long as membrane phase transition temperatures remained below a key threshold.

3.3. Thermotropic and lyotropic phase transitions

Based on our results on membrane phase transition and lipid order, we investigated the possibility of improving cell viability during dehydration by altering the drying and rehydration conditions. Previous results indicated that during drying at 30 °C the cell membrane underwent lyotropic phase change from l.c. to gel phase. Similarly, upon re-hydration, the membrane experienced a lyotropic phase change, this time transitioning from gel to l.c. phase. In order to explore the effect of lyotropic phase change, *G. sulfurreducens* samples were dried at different temperatures (30 or 5 °C). The cells dried at 30 °C for 45 min experienced lyotropic phase transition as indicated by their T_m > 30 °C (Fig. 2B). However, the cells dried at 5 °C experienced a thermotropic phase change from l.c. to gel phase during cooling (prior to dehydration). Therefore, when dried, these cells were already in the

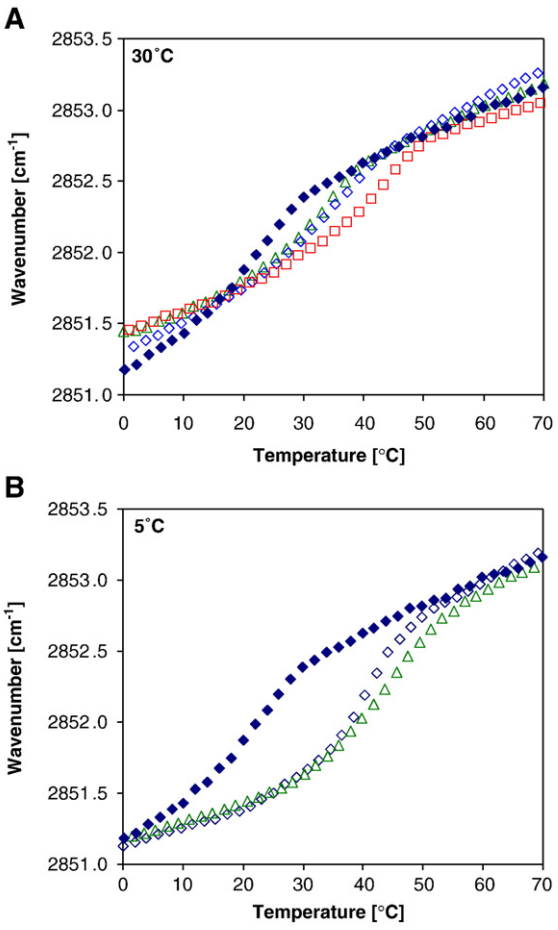

Fig. 4. Comparison of the membrane phase behavior of *G. sulfurreducens* dried in growth medium containing different concentrations of sucrose. (A) Change in νCH_2 maxima with temperature for *G. sulfurreducens* in growth medium (hydrated control) (♦), dried in growth medium (◊), dried in growth medium+6.75% w/w sucrose (△) and dried in growth medium+13.5% w/w sucrose (□) (samples were dried at 30 °C for 45 min). (B) Change in νCH_2 maxima with temperature for *G. sulfurreducens* in growth medium (hydrated control) (♦), dried in growth medium (◊) and dried in growth medium+6.75% w/w sucrose (△) (samples were dried at 5 °C for 45 min) (Representative data sets).

gel phase and lyotropic phase transition was completely eliminated in the 5 °C group.

3.3.1. Membrane structure change with lyotropic phase change (T_d=30 °C group)

Temperature ramp FTIR spectroscopy analysis revealed that T_m values for the cells dried in the growth medium (0% sucrose) (T_m=35±1.5 °C) and growth medium+6.75% sucrose (T_m=35±4 °C) were similar while those dried in growth medium+13.5% sucrose were higher (T_m=40±1 °C) (Fig. 4A). All dried samples had higher wavenumber values at 0 °C as compared to the hydrated *G. sulfurreducens* sample, indicating reduced membrane lipid order at this temperature [47], especially for the cells dried in the presence of sucrose, reflecting a change in the membrane structure (see Discussion). A comparison of lipid order at the l.c. state at higher temperatures for growth medium (0% sucrose) and growth medium+6.75% sucrose was not performed due to increased drying at the higher temperatures. The phase transition cooperativities (ξ) of the dried samples were not significantly different.

3.3.2. Membrane structure change with thermotropic phase change (T_d=5 °C group)

When cells were dried at 5 °C (preventing a lyotropic phase change during drying), *G. sulfurreducens* samples dried in growth medium and growth medium+6.75% sucrose solutions also had high average T_m values. T_m values for the cells dried in growth medium (0% sucrose) (T_m=40±6 °C) and growth medium+6.75% sucrose (T_m=42±3.5 °C) were similar. Unlike the cells dried at 30 °C, wavenumber values at 0 °C were similar to hydrated *G. sulfurreducens* cells indicating that membrane lipid order did not decrease during dehydration at 5 °C. Membrane phase transition cooperativity of *G. sulfurreducens* dried in growth medium (ξ=0.077±0.005 cm^{-1}/°C) was greater than that of *G. sulfurreducens* dried in growth medium containing 6.75% sucrose (ξ=0.059±0.001 cm^{-1}/°C) (Table 2). Cooperativity of *G. sulfurreducens* dried in growth medium+6.75% sucrose at 5 °C was slightly higher than that of *G. sulfurreducens* dried in growth medium at 30 °C (Table 2). These results showed that dehydration at 5 °C created significantly different conditions in the cell membranes.

3.4. Effects of thermotropic and lyotropic membrane phase transitions on post-rehydration viability

Effects of various drying and rehydration conditions on post-rehydration viability of *G. sulfurreducens* were also examined (Fig. 5). *G. sulfurreducens* suspended in growth medium (containing up to 13.5% sucrose) were dried at 5 or 30 °C, and re-hydrated at 5, 30 or 40 °C. These conditions were chosen based on FTIR spectroscopy and viability data shown above to investigate the combined effects of sucrose and thermotropic and lyotropic phase transitions on the viability *G. sulfurreducens* following dehydration and rehydration.

3.4.1. Effect of the lyotropic phase change during re-hydration on G. sulfurreducens viability

Viability measurements were performed with *G. sulfurreducens* samples dried for 45 min in the growth medium containing 0, 6.75, and 13.5% sucrose at T_d=30 °C and 65% RH. Re-hydration was performed at T_r=30 °C to ensure that *G. sulfurreducens* underwent lyotropic membrane phase transition during re-hydration, or at T_r=5 °C or T_r=40 °C to eliminate lyotropic phase transition. In the preliminary experiments, we verified that *G. sulfurreducens* (which has maximum growth rate at 35 °C) could survive incubation at 40 °C without loss of viability (data not shown).

Viability of *G. sulfurreducens* dried at T_d=30 °C in the growth medium (0% sucrose) for T_r=30 °C and T_r=40 °C (Fig. 5) were not significantly different (P=0.159). Similar results, showing a lack of significant change in viability due to re-hydration at a higher temperature, were obtained for the 6.75% sucrose (P=0.262) and the 13.5% sucrose (P=0.242) samples (Fig. 5). The lack of a significant improvement or protective effect due to re-hydration at temperatures higher than T_m indicated that a lyotropic phase transition during re-hydration did not affect viability and the reduction in viability was actually due to lyotropic phase transition during dehydration. This was also confirmed with low temperature experiments presented below.

For T_r=30 °C, viability of *G. sulfurreducens* dried at T_d=30 °C in the growth medium (0% sucrose) and in the growth medium containing 6.75% sucrose were not significantly different for (P=0.1540), which was similar to the previous results obtained with rapid hyperosmotic shock (Table 1). At T_d=30 °C viability of the cells dried in the growth medium containing 13.5% sucrose was significantly lower (P=0.0316)

Table 2
Effect of Drying Conditions on Membrane Phase Transition Cooperativity

Growth Medium (Control Group)	Growth Medium (T_d=30°C group)	Growth Medium (T_d=5°C group)	Growth Medium±6.75% w/w sucrose (T_d=5°C group)
0.058± 0.001 $cm^{-1}°C^{-1}$	0.055± 0.002 $cm^{-1}°C^{-1}$	0.077± 0.005 $cm^{-1}°C^{-1}$	0.059± 0.001 $cm^{-1}°C^{-1}$

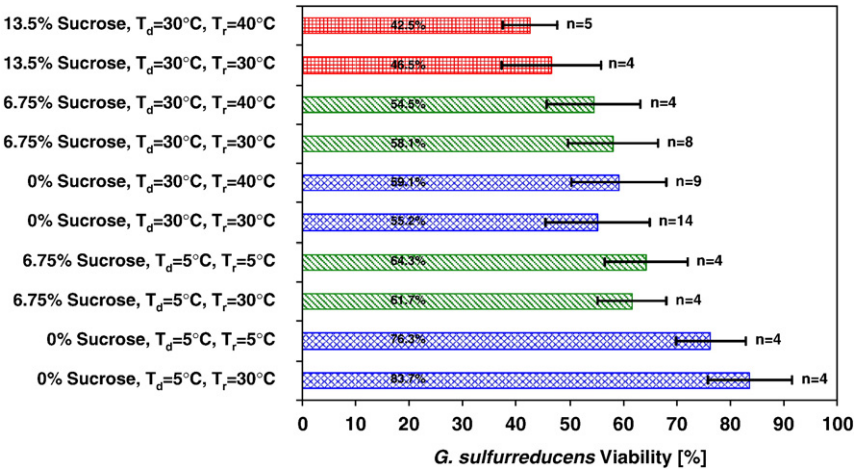

Fig. 5. Post re-hydration viability of *G. sulfurreducens* dried for 45 min at 65% RH. (T_d: drying temperature, T_r: re-hydration temperature) ($n>3$)

than those dried in the growth medium containing 6.75%, also similar to what was observed in hyperosmotic shock experiments. For T_r=40 °C, viability of the cells dried at T_d=30 °C in the growth medium (0% sucrose) and in the growth medium containing 6.75% sucrose were not significantly different (P=0.356). However, viability at both conditions were significantly higher than that obtained in the growth medium containing 13.5% sucrose (P=0.001 and P=0.029, respectively).

3.4.2. Effect of the lyotropic phase change during dehydration on G. sulfurreducens viability

In order to eliminate lyotropic phase transition during dehydration, cells were initially cooled (without drying) down to 5 °C, where they were isothermally dried. Re-hydration was performed either at T_r=5 or 30 °C (Fig. 5). Viability of *G. sulfurreducens* dried in growth medium at 5 °C was significantly higher than all of the other conditions examined ($P\leq$0.001). This demonstrated the pronounced effect of avoiding lyotropic phase transition during dehydration. However, for T_d=5 °C there was a significant decrease in viability for the growth medium+6.75% sucrose group ($P\leq$0.029) when compared to *G. sulfurreducens* dried in growth medium (Fig. 5), showing that higher osmotic stress was detrimental even when the membrane was already in the gel phase. There was no statistically significant difference between the T_r=5 °C and T_r=30 °C groups at T_d=5 °C for either the growth medium (P=0.0986) or growth medium+6.75% sucrose (P=0.309) solutions, once again proving that membrane phase change was not detrimental for *G. sulfurreducens* during re-hydration.

4. Discussion

In certain biotechnological applications bacteria need to be stably encapsulated in a matrix and preserved for transportation and storage. During encapsulation cells are often exposed to high osmotic stresses due to polymerization of the matrix material and the presence of carbohydrates in the surrounding medium. Carbohydrates serve two purposes; they are used to adjust the porosity of the matrix and to protect the bacteria against freezing and dehydration damage. In this study, we performed spectroscopic analysis to understand the mechanisms of hyperosmotic damage to *G. sulfurreducens* in order to develop methodologies that maximize viability during, a) encapsulation in bioreactive coatings, and b) preservation of the encapsulated bacteria in a dehydrated state. Knowing that the cell membrane is a primary site of osmotic injury [26,27], we have focused our attention to the osmotically-induced alterations in the cell membrane. Temperature ramp FTIR spectroscopy analysis was used to provide a measure of the structure, lipid order, and the thermodynamic transitions of the membrane. We have conclusively shown that the information collected through FTIR analysis could be used to design techniques to maximize the viability of the bacteria during encapsulation and preservation.

We have conducted controlled dehydration and hyperosmotic shock experiments with *G. sulfurreducens*. With increasing concentration of sucrose in the extracellular solution, viability of the bacteria decreased. Extracellular sucrose altered the bacteria membrane structure, however, it neither inhibited the increase of T_m nor protected the bacteria against dehydration. It is well known that to protect the membrane structure sugars should be present on both sides of the cell membrane [49]. Hence, the lack of dehydration protection by sucrose was not surprising. In fact, the presence of extracellular sucrose generated additional hyperosmotic stress, which correlated to a decrease in viability.

The high water affinity of sucrose [50] created a competition for water between the sucrose and the membrane lipids. This high affinity of sucrose for water can be seen by comparing the amount of water per cell and the T_m for *G. sulfurreducens* dried in 0% and 13.5% sucrose solutions for 45 min (T_d=30 °C, RH=65%). Using spectroscopic analysis, it was found that the water content in the 13.5% sucrose samples (Viability: 46.5±9.3%) was on average 80% more than that in the 0% sucrose samples (Viability: 55.2±9.7%). The higher T_m for the 13.5% sucrose sample demonstrated that the extra water was not available to the membrane. The direct correlation between *G. sulfurreducens* viability and its membrane phase transition temperature (Fig. 3) also showed that the determining factor is the availability of the water in the sample, not its quantity. This also shows that when comparing samples, thermogravimetric measurement of sample water content (a commonly utilized method) can be misleading since water content does not give an indication of the hydration level of the membrane.

We have quantified the hyperosmotic stress-induced structural changes in the cellular membrane by the change in the νCH_2 band peak location, which corresponds to the order of the lipid acyl chain. For example, due to higher concentration of sucrose in the medium, membrane lipid order in the l.c. phase of *G. sulfurreducens* suspended in 13.5% and 27% sucrose solutions (Fig. 1) was greater than that in the 0% or 6.75% sucrose solutions. On the other hand, the large decrease in lipid order in the gel phase for *G. sulfurreducens* in the 27% sucrose solution (Fig. 1) can be attributed to the destabilization of the membrane such as membrane rupture as suggested by Beney et al. [30]. Changes reflected by increased fluidity, which encompasses decreased membrane lipid order, have also been observed in *Bradyrhizobium japonicum* (also a gram-negative bacteria) when it was subjected to increased osmotic pressure [30]. In addition to decreased

lipid order of the membrane in the gel phase, decreased cooperativity of the phase transition and increase in T_m were also observed during hyperosmotic shock and slow dehydration.

In general, the effect of hyperosmotic shock on the membrane was marked by changes in the lipid order and phase transition cooperativity (Fig. 1), whereas the effect of slow dehydration was reflected by an increase in T_m and decrease in cooperativity (Fig. 2B). The direct correlation between T_m and viability (Fig. 3) obtained during slow dehydration shows that decreasing water activity is directly responsible for the damage to the cell. The viability of *G. sulfurreducens* dried in the 13.5% sucrose solution (T_d=30 °C; RH=65%) is the same as that in the 27% sucrose solution. However, the pronounced difference in the membrane structure and phase behavior between the two samples illustrates the difference between hyperosmotic shock and slow dehydration. The loss of phase transition cooperativity and low gel phase lipid order in *G. sulfurreducens* in the 27% sucrose sample may indicate the lack of stabilizing interactions among neighboring lipids. These characteristics may be attributed to membrane dissolution and disruption during hyperosmotic shock, whereas the small changes in cooperativity and lipid order observed in the slow dehydrated samples may indicate more subtle structural changes such as membrane fusion and vesiculation. These results point to the existence of two different mechanisms of membrane damage for hyperosmotic shock and slow dehydration.

Exposing *G. sulfurreducens* to slow dehydration stress in the absence of sucrose at temperatures (T_d=5 °C) lower than the membrane phase transition temperature (i.e. eliminating lyotropic phase transition during dehydration) had a significant beneficial effect on the post-rehydration viability (Fig. 5). It also revealed a direct correlation between the membrane structure (indicated by lipid order and cooperativity) and viability. *G. sulfurreducens* dried under these conditions had gel phase membrane lipid order similar to that of the hydrated control sample (Fig. 4B) and also had significantly increased phase transition cooperativity as compared to the hydrated control sample (Table 2) (P=0.016).

The higher phase transition cooperativity of growth medium samples dried at T_d=5 °C (Fig. 4B, Table 2) can be explained by stabilization of the gel phase due to higher gel–gel lipid interaction energies [51]. Cooperativity of a thermotropic gel to l.c. phase transition for a lipid bilayer is dependent on the magnitude of nearest neighbor interaction energy, ε, [51] between the lipid molecules in the gel and l.c. phases as given by:

$$\varepsilon = |2\varepsilon_{\text{l.c.–gel}} - \varepsilon_{\text{gel–gel}} - \varepsilon_{\text{l.c.–l.c.}}|. \quad (1)$$

An increase in the absolute magnitude of ε corresponds to an increase in cooperativity of a thermotropic gel to l.c. phase transition [51]. An increase in ε, and therefore cooperativity, results from an increase in the stability of the gel–gel region interactions as might be expected in the dried state. Therefore, with increasing dehydration, i.e. higher T_m values, an increase in cooperativity is expected for a membrane that retained its original overall structure and organization. Therefore, the higher cooperativity values measured for growth medium samples (T_d=5 °C) indicated that the cell membranes experienced minimal destructive changes when dried under these conditions. The reduction of destructive changes, such as fusion and vesiculation, may be due to the membrane bilayer being energetically less susceptible to fusion events in the gel phase [52].

Even though the damage associated with lyotropic phase change during dehydration could be minimized by drying *G. sulfurreducens* at T_d=5 °C, the additional stress due to presence of extracellular sucrose can still be detrimental. Even the presence of 6.75% sucrose caused a significant decrease in viability (Fig. 5). This was reflected by the significantly lower cooperativity of 6.75% sucrose samples (T_d=5 °C) (P=0.017) as compared to growth medium samples (T_d=5 °C) (Table 2). *G. sulfurreducens* samples slowly dehydrated in 0% and 13.5% sucrose (T_d=30 °C) also did not exhibit increased cooperativity with increased

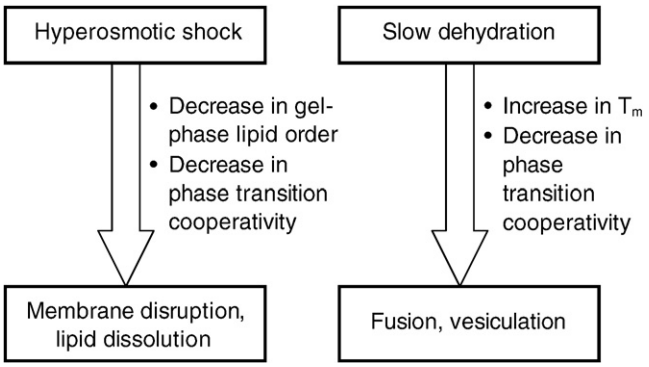

Fig. 6. Schematic showing the two modes of osmotic stress and the respective suggested mechanisms of membrane damage (associated FTIR spectroscopy measurements are also shown).

T_m (T_m>32 °C) due to already existing membrane damage with progressive dehydration (i.e. the increase in membrane damage with T_m beyond a certain threshold offsets the propensity for increased cooperativity.

The results presented here highlight the two main factors of importance during dehydration of *G. sulfurreducens*. The first factor is the existence of two distinct mechanisms of membrane damage dependent on the rate of osmotic stress (Fig. 6). The second factor is that the lyotropic phase transition during drying enhances membrane damage. By considering these two factors it may be possible to design an appropriate procedure to produce coatings with a high percentage of viable *G. sulfurreducens* bacteria.

5. Summary

We determined that *G. sulfurreducens* viability decreased with increasing sucrose concentration in the medium showing that extracellular sucrose acted only as an osmolyte and did not protect *G. sulfurreducens* against the dehydration stress. Depending on the rate of application of the hyperosmotic stress, we identified two different membrane damage mechanisms to be responsible for viability loss in *G. sulfurreducens*. We have also established that the resistance of *G. sulfurreducens* to hyperosmotic stress could be improved when lyotropic phase transition was avoided during dehydration by desiccating the bacteria at a temperature lower than the membrane phase transition temperature. We have therefore distinguished the effects of the lyotropic and thermotropic phase transitions of the cell membrane on the hyperosmotic stress survival of *G. sulfurreducens*.

Acknowledgements

This research was supported by a grant (GIA-20328) from the office of the Dean of the Graduate School of the University of Minnesota. Authors thank Ms. Cally Scherber for her assistance with the FTIR spectroscopy experiments.

References

[1] D.R. Bond, D.R. Lovley, Electricity production by *Geobacter sulfurreducens* attached to electrodes, Appl. Environ. Microbiol. 69 (2003) 1548–1555.
[2] J.R. Lloyd, D.R. Lovley, L.E. Macaskie, Biotechnological application of metal-reducing microorganisms, Adv. Appl. Microbiol. 53 (2003) 85–128.
[3] D.R. Lovley, Dissimilatory Fe (III) and Mn (IV) reduction, Microbiol. Mol. Biol. Rev. 55 (1991) 259.
[4] D.R. Lovley, Microbial fuel cells: novel microbial physiologies and engineering approaches, Curr. Opin. Biotechnol. 17 (2006) 327–332.
[5] M.E. Hernandez, D.K. Newman, Extracellular electron transfer, Cell. Mol. Life Sci. (CMLS) 58 (2001) 1562–1571.
[6] J.D. Wall, L.R. Krumholz, Uranium reduction, Annu. Rev. Microbiol. 60 (2006) 149–166.

[7] M.C. Flickinger, J.L. Schottel, D.R. Bond, A. Aksan, L.E. Scriven, Painting and printing living bacteria: engineering nanoporous biocatalytic coatings to preserve microbial viability and intensify reactivity, Biotechnol. Prog. 23 (2007) 2–17.
[8] M.J. Wilkins, F.R. Livens, D.J. Vaughan, J.R. Lloyd, The impact of Fe (III)-reducing bacteria on uranium mobility, Biogeochemistry 78 (2006) 125–150.
[9] D.R. Bond, D.E. Holmes, L.M. Tender, D.R. Lovley, Electrode-reducing microorganisms that harvest energy from marine sediments, Science 295 (2002) 483–485.
[10] A. Shantaram, H. Beyenal, R. Raajan, A. Veluchamy, Z. Lewandowski, Wireless sensors powered by microbial fuel cells, Environ. Sci. Technol. 39 (2005) 5037–5042.
[11] S. Srikanth, E. Marsili, M.C. Flickinger, D.R. Bond, Electrochemical characterization of *Geobacter sulfurreducens* cells immobilized on graphite paper electrodes, Biotechnol. Bioeng. 99 (2007) 1065–1073.
[12] V.S. Thiagarajan, Z. Huang, L.E. Scriven, J.L. Schottel, M.C. Flickinger, Microstructure of a biocatalytic latex coating containing viable *Escherichia coli* cells, J. Colloid Interface Sc. 215 (1999) 244–257.
[13] O.K. Lyngberg, D.J. Stemke, J.L. Schottel, M.C. Flickinger, A single-use luciferase-based mercury biosensor using *Escherichia coli* HB101 immobilized in a latex copolymer film, J. Ind. Microbiol. Biotech. 23 (1999) 668–676.
[14] O.K. Lyngberg, C.P. Ng, V.S. Thiagarajan, L.E. Scriven, M.C. Flickinger, Engineering the microstructure and permeability of thin multilayer latex biocatalytic coatings containing *E. coli*, Biotechnol. Prog. 17 (2001) 1169–1179.
[15] D.K. Hincha, M. Hagemann, Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms, Biochem. J. 383 (2004) 277–283.
[16] J.H. Crowe, L.M. Crowe, D. Chapman, Preservation of membranes in anhydrobiotic organisms — the role of trehalose, Science 223 (1984) 701–703.
[17] X.H. Liu, A. Aksan, M.A. Menze, S.C. Hand, M. Toner, Trehalose loading through the mitochondrial permeability transition pore enhances desiccation tolerance in rat liver mitochondria, BBA-Biomembranes 1717 (2005) 21–26.
[18] S.B. Leslie, E. Israeli, B. Lighthart, J.H. Crowe, L.M. Crowe, Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying, Appl. Environ. Microbiol. 61 (1995) 3592–3597.
[19] H. Oldenhof, W.F. Wolkers, F. Fonseca, S. Passot, M. Marin, Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: an in situ Fourier transform infrared spectroscopy study, Biotechnol. Prog. 21 (2005) 885–892.
[20] T. Kanias, J.P. Acker, Mammalian cell desiccation: facing the challenges, Cell Preserv. Technol. 4 (2006) 253–277.
[21] M.M. Norris, A. Aksan, K. Sugimachi, M. Toner, 3-*O*-methyl-D-glucose improves desiccation tolerance of keratinocytes, Tissue Eng. 12 (2006) 1–7.
[22] S.J. Webb, Bound Water in Biological Activity, Charles C. Thomas, Springfield, IL, 1965.
[23] J.H. Crowe, J.S. Clegg, Anhydrobiosis, Dowden, Hutchinson, and Ross, Stroudsburg, 1973.
[24] J.H. Crowe, L.M. Crowe, J.F. Carpenter, S.J. Prestrelski, F.A. Hoekstra, P. de Araujo, A.D. Panek, Anhydrobiosis: cellular adaptation to extreme dehydration, Handb. Physiol. 2 (1997) 1445–1477.
[25] J.H. Crowe, J.F. Carpenter, L.M. Crowe, The role of vitrification in anhydrobiosis, Annu. Rev. Physiol. 60 (1998) 73–103.
[26] P.L. Steponkus, Role of the plasma membrane in freezing injury and cold acclimation, Ann. Rev. Plant Physiol. 35 (1984) 543–584.
[27] Y. Mille, L. Beney, P. Gervais, Viability of *Escherichia coli* after combined osmotic and thermal treatment: a plasma membrane implication, BBA-Biomembranes 1567 (2002) 41–48.
[28] L. Beney, Y. Mille, P. Gervais, Death of *Escherichia coli* during rapid and severe dehydration is related to lipid phase transition, Appl. Microbiol. Biotechnol. 65 (2004) 457–464.
[29] Y. Mille, L. Beney, P. Gervais, Magnitude and kinetics of rehydration influence the viability of dehydrated *E. coli* K-12, Biotechnol. Bioeng. 83 (2003) 578–582.
[30] L. Beney, H. Simonin, Y. Mille, P. Gervais, Membrane physical state as key parameter for the resistance of the gram-negative *Bradyrhizobium japonicum* to hyperosmotic treatments, Arch. Microbiol. 187 (2007) 387–396.
[31] J. Milhaud, New insights into water–phospholipid model membrane interactions, BBA-Biomembranes 1663 (2004) 19–51.
[32] J.F. Nagle, Theory of the main lipid bilayer phase transition, Ann. Rev. Phys. Chem. 31 (1980) 157–196.
[33] D.L. Melchior, J.M. Steim, Thermotropic transitions in biomembranes, Ann. Rev. Biophys. Bioeng. 5 (1976) 205–238.
[34] J.H. Crowe, L.M. Crowe, F.A. Hoekstra, Phase-transitions and permeability changes in dry membranes during rehydration, J. Bioenerg. Biomembr. 21 (1989) 77–91.
[35] L.M. Hays, J.H. Crowe, W. Wolkers, S. Rudenko, Factors affecting leakage of trapped solutes from phospholipid vesicles during thermotropic phase transitions, Cryobiology 42 (2001) 88–102.
[36] F.M. Harris, K.B. Best, J.D. Bell, Use of laurdan fluorescence intensity and polarization to distinguish between changes in membrane fluidity and phospholipid order, BBA-Biomembranes 1565 (2002) 123–128.
[37] D. Chapman, J. Urbina, K.M. Keough, Biomembrane phase transitions: studies of lipid–water systems using differential scanning calorimetry, J. Biol. Chem. 249 (1974) 2512–2521.
[38] W.F. Wolkers, L.M. Crowe, N.M. Tsvetkova, F. Tablin, J.H. Crowe, In situ assessment of erythrocyte membrane properties during cold storage, Mol. Membr. Biol. 19 (2002) 59–65.
[39] F.A. Hoekstra, J.H. Crowe, L.M. Crowe, Effect of sucrose on phase-behavior of membranes in intact pollen of *Typha-Latifolia* L, as measured with Fourier-transform infrared-spectroscopy, Plant Physiol. 97 (1991) 1073–1079.
[40] C. Laroche, H. Simonin, L. Beney, P. Gervais, Phase transitions as a function of osmotic pressure in *Saccharomyces cerevisiae* whole cells, membrane extracts and phospholipid mixtures, BBA-Biomembranes 1669 (2005) 8–16.
[41] E.Z. Drobnis, L.M. Crowe, T. Berger, T.J. Anchordoguy, J.W. Overstreet, J.H. Crowe, Cold shock damage is due to lipid phase-transitions in cell-membranes — a demonstration using sperm as a model, J. Exp. Zool. 265 (1993) 432–437.
[42] G. Anderle, R. Mendelsohn, Thermal denaturation of globular proteins. Fourier transform-infrared studies of the amide III spectral region, Biophys. J. 52 (1987) 69–74.
[43] E. Goormaghtigh, J.M. Ruysschaert, V. Raussens, Evaluation of the information content in infrared spectra for protein secondary structure determination, Biophys. J. 90 (2006) 2946–2957.
[44] C.A. Morgan, N. Herman, P.A. White, G. Vesey, Preservation of micro-organisms by drying; a review, J. Microbiol. Methods 66 (2006) 183–193.
[45] K. Brandenburg, U. Seydel, Infrared spectroscopy of glycolipids, Chem. Phys. Lipids 96 (1998) 23–40.
[46] H. Susi, D.M. Byler, Protein structure by Fourier transform infrared spectroscopy: second derivative spectra, Biochem. Biophys. Res. Commun. 115 (1983) 391–397.
[47] W.F. Wolkers, S.A. Looper, R.A. Fontanilla, N.M. Tsvetkova, F. Tablin, J.H. Crowe, Temperature dependence of fluid phase endocytosis coincides with membrane properties of pig platelets, BBA-Biomembranes 1612 (2003) 154–163.
[48] M. Potts, Desiccation tolerance of prokaryotes, Microbiol. Rev. 58 (1994) 775–805.
[49] L.M. Crowe, J.H. Crowe, A. Rudolph, C. Womersley, L. Appel, Preservation of freeze-dried liposomes by trehalose, Arch. Biochem. Biophys. 242 (1985) 240–247.
[50] M.J. Blandamer, J. Engberts, P.T. Gleeson, J.C.R. Reis, Activity of water in aqueous systems; a frequently neglected property, Chem. Soc. Rev. 34 (2005) 440–458.
[51] R.L. Biltonen, A statistical-thermodynamic view of cooperative structural changes in phospholipid bilayer membranes: their potential role in biological function, J. Chem. Thermodyn. 22 (1990) 1–19.
[52] D.P. Siegel, The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion, Biophys. J. 76 (1999) 291–313.