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Moisture sorption characteristics of freeze-dried human platelets*

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Abstract: Freeze-drying is a promising method for a long-term storage of human platelets. The moisture sorption characteristics of freeze-dried human platelets (FDHPs) were studied in this paper. The moisture sorption isotherms of FDHPs and freeze-dried lyophilization buffer (FDLB) were measured at 4, 25, and 37 °C. The experimental data were fitted to Brunauer-Emmett-Teller (BET) and Guggenheim-Anderson-de Boer (GAB) equations. There were no significant statistical differences ($P>0.05$) between the sorption characteristics of FDHPs and FDLB at 4 and 25 °C, while FDHPs absorbed more water at 37 °C. The net isosteric heat of sorption was derived. The heat for FDHPs showed an abnormal negative value at low moisture contents when 25 and 37 °C data were used. Dynamic sorption experiments were carried out at 25 °C with environmental water activity controlled at 0.75, 0.85, and 0.90. The moisture diffusion coefficient was fitted to be 8.24×10^{-12} m^2/s when experimental data at initial time were used. These results would be helpful in choosing prehydration and storage condition for FDHPs.

Key words: Human platelets, Freeze-drying, Sorption, Moisture
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1 Introduction

Freeze-drying or lyophilization, as a preservation method allowing materials to be stored at room temperature, has many advantages such as keeping of heat-sensitive components, no need for cooling equipment, low storage cost, and convenient transportation (Sitaula and Bhowmick, 2006). It has been widely employed in the field of preservation of foods, pharmaceuticals, and biological materials.

Human platelets are widely used in transfusion medicine due to their adhesive, hemostatic, and procoagulant properties (Bode and Read, 2000). However, seasonal and occasional shortages of human platelet supply happen as unused human platelets have to be discarded. The present method for a long-term storage of human platelets is cryopreservation, which demands expensive equipments, thus making storage of large quantities of platelets not possible. The freeze-drying of platelets is a promising choice that appears in recent years. Wolkers *et al.* (2001) found that platelets loaded with trehalose were successfully freeze-dried with a survival rate of 85%. Prehydration in water vapor was proved to be a beneficial step, which led to better morphology for hydrated freeze-dried human platelets (FDHPs) similar to that of fresh platelets. Fan *et al.* (2009) tried varied prehydration conditions for 1 ml FDHPs held in vial and obtained the optimal prehydration time. The physical mechanisms underlying the prehydration process, namely the moisture sorption characteristics of FDHPs, were not elucidated in these studies, and thus the prehydration conditions for the other cases still remain unclear.

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For the storage stability of the freeze-dried products, it is important to get the knowledge of their moisture sorption isotherms, where the equilibrium moisture contents can be predicted from the water activities. A large number of relevant works have been done for foods (McLaughlin and Magee, 1998; McMinn and Magee, 1999; Gabas *et al*., 2007; Omar and Roos, 2007; Wang *et al*., 2008; Fabra *et al*., 2009) and pharmaceutical products (Zeng *et al*., 2001; Columbano *et al*., 2002; van Drooge *et al*., 2006; Salnikova *et al*., 2008). While there was little research about the sorption characteristics of freeze-dried cells, except that Sitaula and Bhowmick (2006) studied the moisture sorption characteristics of trehalose-phosphate buffer solution (PBS) mixtures, which were often used as components of lyophilization buffer for cell freeze-drying.

The objective of this study was to quantify the moisture sorption characteristics of FDHPs, including equilibrium and dynamic characteristics. The Brunauer-Emmett-Teller (BET) and Guggenheim-Anderson-de Boer (GAB) equations were used to fit the equilibrium data. Three temperature levels were considered for the estimation of the isosteric heat of sorption. The experiments with freeze-dried lyophilization buffer (FDLB) were also carried out for comparison.

2 Materials and methods

2.1 Preparation of freeze-dried samples

The trehalose loading buffer [100 mmol/L NaCl, 10 mmol/L KCl, 10 mmol/L ethylene glycol tetraacetic acid (EGTA), 10 mmol/L imidazole, and 50 mmol/L trehalose; pH 6.8] and the lyophilization buffer [9.5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 142.5 mmol/L NaCl, 4.8 mmol/L KCl, and 1 mmol/L $MgCl_2$ as the base solution, with the lyoprotectants including 200 g/L trehalose and 10 g/L bovine serum albumin (BSA)] were prepared in advance and stored at 4 °C.

Platelet concentrates [platelet-rich plasma (PRP)] of healthy volunteers provided by the Blood Center of Zhejiang Province (China), were stored on a flatbed shaker overnight at 22 °C before use. PRP was centrifuged at 2000 r/min for 10 min and washed twice in physiological saline.

Washed platelets were resuspended in the trehalose loading buffer with a concentration of about 1×10^9 platelets/ml, and then incubated in a 37 °C water bath for 4 h to load trehalose into cells. After incubation, the platelet suspensions were centrifuged at 2000 r/min for 3 min and resuspended in the lyophilization buffer with a concentration of about 1×10^9 platelets/ml.

A total of 1 ml platelet suspension was filled into glass vials (inner diameter 19 mm) for lyophilization. A total of 1 ml lyophilization buffer alone was also lyophilized at the same time for comparison.

The vials were first cooled in a cryo-refrigerator at −60 °C for 2 h, and then were freeze-dried using a laboratory-scale freeze-drier (Labconco, USA) of which the cold condenser could reach −80 °C. The vacuum pressure was kept at about 1 Pa. The primary drying lasted for 16 h while the shelf temperature was maintained at −40 °C. For the secondary drying the shelf temperature was ramped to 20 °C at a rate of 0.2 °C/min and held for another 24 h. After freeze-drying, the samples were sealed with rubber stoppers automatically.

The water contents of freeze-dried samples were measured by a thermogravimetry method using Q600 Simultaneous DSC-TGA (TA Instruments, USA). About 5 mg of powders were placed on the sample pans and heated from room temperature to 120 °C at a heating rate of 10 °C/min.

2.2 Equilibrium moisture sorption measurement

Moisture sorption isotherms of FDHPs and FDLB were obtained using the static gravimetric method. Seven saturated salt solutions were prepared to generate a range of water activities from 0.11 to 0.88, respectively (Table 1) (Greenspan, 1977). Accurately weighed samples (30–50 mg) by an electronic balance (precision 0.1 mg; Mettler AE200, Switzerland) were held in small plastic pans and placed into a sealed container, as shown in Fig. 1. The temperature of the water bath was held constant. Three temperature levels (4, 25, and 37 °C) were tested. Three samples were repeated for each case and blank pans were used as controls. The weights of the samples and controls were measured gravimetrically once a week, until equilibrium was reached.

Table 1 Water activities of saturated salt solutions at different temperatures

Saturated salt solution	Water activity		
	4 °C	25 °C	37 °C
LiCl	0.13	0.11	0.11
CH_3COOK	0.27	0.23	0.20
$MgCl_2$	0.34	0.33	0.32
K_2CO_3	0.43	0.43	0.43
$Mg(NO_3)_2$	0.59	0.53	0.49
NaCl	0.76	0.75	0.75
KCl	0.88	0.84	0.83

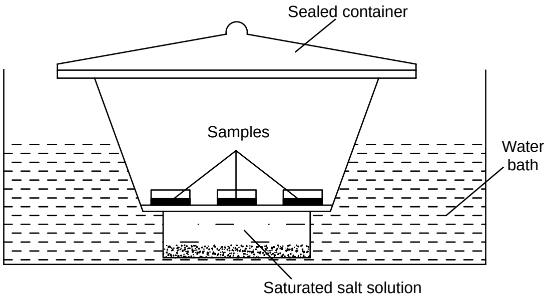

Fig. 1 Samples held at a specific water activity and temperature

2.3 Dynamic moisture sorption measurement

The dynamic moisture sorption experiments were performed at (25±1) °C at relative humidity (RH) of 75%, 85%, and 90% using a set-up as shown in Fig. 2. The vial freeze-dried sample was suspended in a specimen chamber, whose weight was monitored by an electronic balance (precision 1 mg; Mettler AL104, Switzerland). Dry nitrogen gas and saturated vapor flows were mixed to generate the required humidity. The temperature was measured with a PT100 thermistor of which the precision was 0.2 °C. The relative humidity was measured with an RH sensor (ALRE, FF-K1, Germany) of which the precision was ±3% RH. The weight of the sample, the RH and temperature of the specimen chamber were recorded by the computer instantaneously.

2.4 Theoretical basis

2.4.1 Equation of moisture sorption isotherm

Among a large number of physical models proposed in the literature describing the moisture sorption isotherms, BET and GAB equations were most widely used. They were chosen to model the sorption behaviors of FDHPs and FDLB.

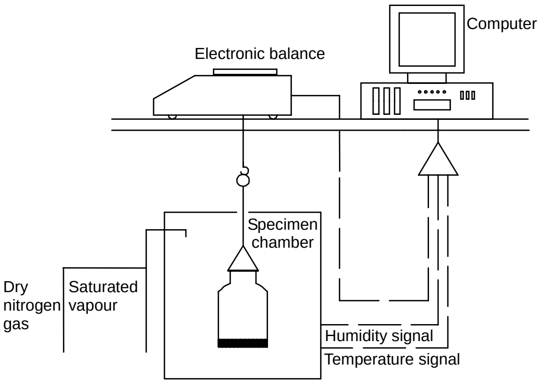

Fig. 2 Set-up for dynamic moisture sorption measurement

The BET equation could give a better interpretation of Types II and III sorption isotherms (Al-Muhtaseb *et al.*, 2002). It provides a theoretical estimation of the monolayer value of moisture absorbed on the surface. In most cases, the BET description is merely appropriate over the lower range of water activities of 0.10–0.40. The equation is generally presented in the following form:

$$M_e = \frac{M_m C a_w}{(1-a_w)\left(1+(C-1)a_w\right)}, \tag{1}$$

where M_e is the moisture content adsorbed per dry weight, a_w is water activity, M_m is a constant representing the moisture content adsorbed as a monomolecular layer (or corresponding to the amount of water needed to surround the material surface with just one layer of water molecules), and C is a constant related to the heat liberated in the monolayer sorption process.

The GAB equation is a semi-theoretical sorption model and is more versatile compared with the BET equation. It is applicable in the water activity range of 0.10–0.90 (McMinn and Magee, 1997; Timmermann *et al.*, 2001). The GAB equation is given as:

$$M_e = \frac{M_{mG} C_G K_G a_w}{(1-K_G a_w)\left(1+(C_G-1)K_G a_w\right)}, \tag{2}$$

where M_{mG} is a constant representing the monolayer moisture content, and C_G and K_G are constants related to the interaction between the first layer and further layers of water molecules at the sorption sites.

2.4.2 Net isosteric heat of sorption

The net isosteric heat of sorption is defined as the difference between the total heat of sorption and the vaporization heat of water at the system temperature. It is considered as an indication of the intermolecular forces between the sorption sites and the moisture content. Based on the thermodynamic principles, it can be determined from the following equation (McMinn and Magee, 1999; Al-Muhtaseb *et al*., 2002; Burnett *et al*., 2006; Gabas *et al*., 2007; Sinija and Mishra, 2008):

$$q_{\mathrm{st}}=Q_{\mathrm{st}}-\lambda=R\left.\frac{\partial\left|\ln a_{\mathrm{w}}\right|}{\partial\left|1/T\right|}\right|_{M_{\mathrm{e}}},\tag{3}$$

where q_{st} is the net isosteric heat of sorption at temperature T and constant moisture content M_{e}, Q_{st} is the total heat of sorption, λ is the vaporization heat of water, and R is the universal gas constant.

If the water activities ($a_{\mathrm{w}1}$ and $a_{\mathrm{w}2}$) corresponding to the same moisture content M_{e} at two temperatures (T_1 and T_2) are known, then the isosteric heat of sorption can be estimated via (Burnett *et al*., 2006; Gabas *et al*., 2007; Sinija and Mishra, 2008)

$$q_{\mathrm{st}}=-R\left.\frac{\ln(a_{\mathrm{w}2}/a_{\mathrm{w}1})}{(1/T_2)-(1/T_1)}\right|_{M_{\mathrm{e}}}.\tag{4}$$

2.4.3 Fick's second law of diffusion

The shapes of FDHPs and FDLB in a glass vial can be treated as a cylinder of which the diameter is 19 mm and the height is 3.5 mm. The following assumptions are made to model the dynamic moisture sorption process of the freeze-dried sample (Perrin *et al*., 1996; Guillard *et al*., 2003; 2004): (1) the moisture diffusion obeys the Fick's second law of diffusion; (2) the sample is homogeneous with constant diffusion coefficient; (3) the sorption process is a much faster process compared with the diffusion process; and, (4) the external resistance to moisture transfer at the sample interface can be neglected. Then the dynamic moisture sorption process can be described by the 1D diffusion equation as follows:

$$\frac{\partial M}{\partial t}=\frac{\partial}{\partial x}\left(D_{\mathrm{eff}}\frac{\partial M}{\partial x}\right),\tag{5}$$

where M is the moisture content at time t and location x, and D_{eff} is the diffusion coefficient.

For the initial stage of diffusion, the moisture mass gain can be well approximated by the following formula (Crank, 1975):

$$\frac{M_t-M_0}{M_{\mathrm{e}}(a_{\mathrm{w}})-M_0}=\frac{4}{l}\left(\frac{D_{\mathrm{eff}}t}{\pi}\right)^{1/2},\tag{6}$$

where M_t is the average moisture content at time t, M_0 is initial moisture content, $M_{\mathrm{e}}(a_{\mathrm{w}})$ is the equilibrium moisture content at water activity a_{w}, determined by the GAB equation, a_{w} is the water activity of the surrounding atmosphere, and l is the thickness of the freeze-dried sample.

3 Results and discussion

3.1 Moisture sorption isotherm

The equilibrium moisture contents of FDHPs and FDLB corresponding to various water activities at three temperature levels are shown in Table 2. Two-tailed P values using the student t-test for comparing FDHPs and FDLB are also listed. The overall trend is that the moisture content increased with increasing water activity. However, at some points, higher water activity led to lower moisture content (McLaughlin and Magee, 1998; Sitaula and Bhowmick, 2006). Statistical differences between the equilibrium moisture contents of FDHPs and FDLB at the same water activity were observed when significance was assessed as $P\leq 0.05$, especially in the case of 37 °C.

The discrepancies between FDHPs and FDLB may be attributed to the presence of the additional platelets. In the lyophilization suspension, the total volume of the cells amounted to be about 1% (v/v) of the solution volume (Michelson, 2007). While after freeze-drying, this percentage was estimated to over 5% (v/v), which was large enough to have impacts on the overall moisture sorption characteristics. The obvious differences observed at 37 °C might be correlated with the changes of membrane physical state of the platelet cells (Wolkers *et al*., 2003), which resulted that more moisture vapor has the chance to enter the intracellular space.

The experimental data in Table 2 were fitted to the BET and GAB equations. Table 3 listed the BET and GAB parameters, as well as those reported by Sitaula and Bhowmick (2006), who studied the moisture sorption isotherms of freeze-dried trehalose-PBS mixtures at (22±3) °C. Figs. 3 and 4 gave the data and the fitted BET and GAB curves. The BET curves were obtained by applying to the equilibrium moisture content data of water activity ≤0.40, while the GAB curves gave appropriate approximations over the whole water activity range. It should be noticed that the isotherms of FDHPs and FDLB are of Type II, exhibiting a sigmoidal curve at low water activities. This type of isotherm was also followed by moisture sorption isotherms of carbohydrate, sugars and their mixtures with salts or proteins (McMinn and Magee, 1997; Pérez-Alonso *et al*., 2006; Sitaula and Bhowmick, 2006; Omar and Roos, 2007; Stasiak and Jamroz, 2008). Compared with Type III isotherm, the corresponding water activity of Type II isotherm is smaller for the same moisture content, which indicates less molecular mobility and facilitates the desiccation preservation of cells (Sitaula and Bhowmick, 2006).

Table 2 Equilibrium moisture contents for FDHPs and FDLB at 4, 25, and 37 °C

Temperature (°C)	Water activity	Moisture content[a]		P^b
		FDHPs	FDLB	
4	0.13	0.0597±0.0068	0.0665±0.0072	0.299
	0.27	0.0961±0.0208	0.0892±0.0063	0.610
	0.34	0.1198±0.0103	0.1279±0.0174	0.524
	0.43	0.1203±0.0058	0.1108±0.0114	0.270
	0.59	0.1368±0.0042	0.1438±0.0090	0.297
	0.76	0.2457±0.0015	0.2220±0.0123	0.030
	0.88	0.3673±0.0086	0.3151±0.0207	0.016
25	0.11	0.0542±0.0055	0.0538±0.0041	0.921
	0.23	0.0998±0.0097	0.0896±0.0096	0.264
	0.33	0.0915±0.0062	0.0778±0.0040	0.033
	0.43	0.0816±0.0051	0.0822±0.0005	0.895
	0.53	0.1161±0.0115	0.1169±0.0008	0.930
	0.76	0.2496±0.0049	0.2134±0.0082	0.003
	0.84	0.2924±0.0075	0.2659±0.0088	0.017
37	0.11	0.0588±0.0171	0.0304±0.0068	0.055
	0.20	0.1134±0.0024	0.0874±0.0074	0.004
	0.32	0.1139±0.0028	0.0791±0.0048	0.001
	0.43	0.1128±0.0055	0.0871±0.0048	0.004
	0.49	0.1131±0.0086	0.0834±0.0032	0.005
	0.75	0.2106±0.0105	0.1863±0.0128	0.063
	0.83	0.2730±0.0034	0.2422±0.0172	0.039

n=3; [a] w/w, dry basis; [b] Significance was assessed as $P \le 0.05$

Table 3 Estimated parameters of BET and GAB models

Sample	Temp. (°C)	Trehalose/PBS[a]	BET model			GAB model			
			M_m	C	R^2	M_{mG}	C_G	K_G	R^2
FDHPs	4	~20:1	0.0804	16.77	0.9700	0.0762	31.48	0.90	0.9891
	25	~20:1	0.0748	20.40	0.9300	0.0688	28.43	0.92	0.9644
	37	~20:1	0.0751	54.15	0.8860	0.0758	33.58	0.86	0.9419
FDLB	4	~20:1	0.0751	30.07	0.9300	0.0786	36.27	0.85	0.9880
	25	~20:1	0.0614	44.82	0.9200	0.0682	27.54	0.89	0.9805
	37	~20:1	0.0589	19.36	0.8300	0.0587	20.10	0.91	0.9400
Freeze-dried trehalose-PBS mixtures[b]	22±3	Pure trehalose	0.0740	30.60		0.0780	19.90	0.99	
	22±3	20:1	0.0790	21.90		0.0780	21.90	1.00	
	22±3	10:1	0.0920	33.40		0.1080	19.30	0.95	

[a] v/v; [b] Data from Sitaula and Bhowmick (2006)

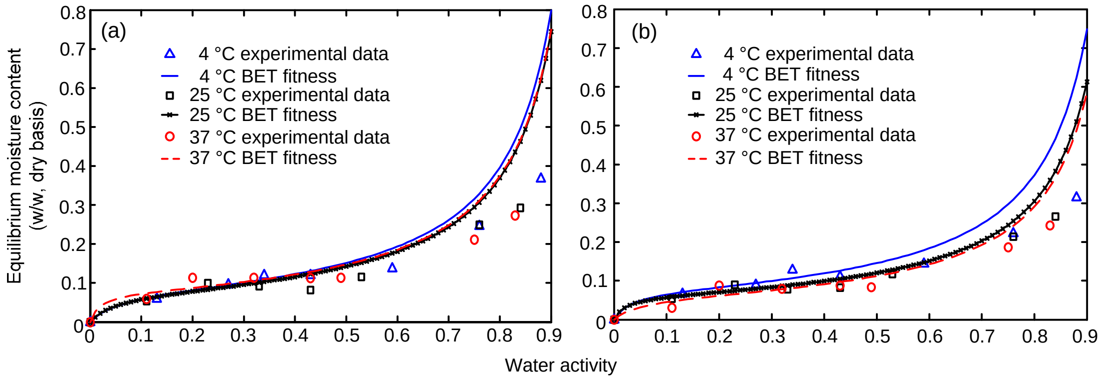

Fig. 3 Equilibrium moisture contents and fitted BET curves of FDHPs (a) and FDLB (b) at different temperatures

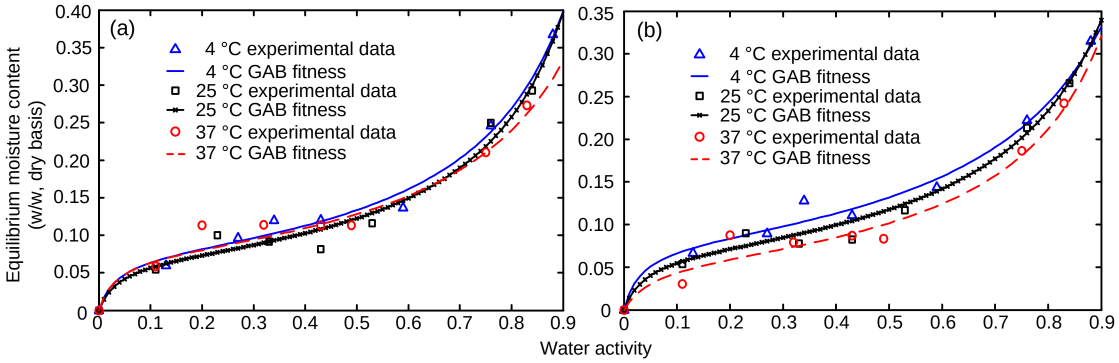

Fig. 4 Equilibrium moisture contents and fitted GAB curves of FDHPs (a) and FDLB (b) at different temperatures

The value of the monolayer moisture content (M_m) is important as it indicates the amount of water strongly adsorbed at the material surface, and is often considered as the critical value to assure material stability (McMinn and Magee, 1997; Sitaula and Bhowmick, 2006). Values of M_m of FDLB decreased from 0.0815 to 0.0587 with increasing temperature from 4 to 37 °C, while M_m of FDHPs did not follow this similar tendency and remained almost unchanged when temperature increased from 25 to 37 °C.

C_G is related to the magnitude of difference between the chemical potentials of monolayer water molecule and multilayer water molecule (McMinn and Magee, 1997; Sitaula and Bhowmick, 2006). The C_G values of FDLB decreased as temperature increased, which is in accordance with the usual assumption that strong adsorbent-adsorbate interactions are favored at lower temperatures (Pérez-Alonso *et al.*, 2006; Gabas *et al.*, 2007). For FDHPs, the values of C_G did not exhibit the same tendency, but showed a maximum at 37 °C.

Generally, the equilibrium moisture contents tend to decrease with increasing temperature at constant water activity (McMinn and Magee, 1997; 1999; 2003; McLaughlin and Magee, 1998; Al-Muhtaseb *et al.*, 2002; Sinija and Mishra, 2008), but totally opposite trend or crossing behavior at high water activity was also observed (Al-Muhtaseb *et al.*, 2002; Pérez-Alonso *et al*. 2006; Gabas *et al.*, 2007). These phenomena were explained by occurrence of different adsorption mechanism or increase of sugar solubility with increasing temperature at water activity above 0.7 for products with high sugar content. Here crossing phenomena could be observed for the isotherms of both FDHPs and FDLB as shown in Figs. 3 and 4, which may be explained by the fact that trehalose is a disaccharide and the presence of platelets.

3.2 Net isosteric heat of sorption

The knowledge of isosteric heat of sorption not only is beneficial for the design of dehydration process, but also provides indication of the amount of 'bound' water existed in the material (Al-Muhtaseb *et al.*, 2002; McMinn and Magee, 2003). The net isosteric heats of sorption q_{st} for FDHPs and FDLB were calculated by applying Eq. (4) to the sorption isotherm data fitted by GAB equations. Two groups calculated using sorption data between 4 and 25 °C and between 25 and 37 °C, respectively, are presented in Fig. 5. For FDLB, q_{st} decreased as the moisture content increased except a peak appeared at a very low moisture content. When the moisture content was larger than 0.30 (w/w, dry basis), q_{st} approached zero, meaning that the limit of bound water was reached. The maximum of q_{st} at moisture content of 0.05 (w/w, dry basis) was also observed by Pérez-Alonso *et al.* (2006) for the moisture sorption of gum Arabic and mesquite gum, which may be associated with the swelling of carbohydrate polymer matrix.

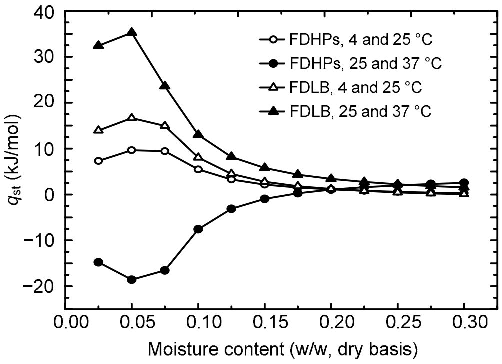

Fig. 5 Net isosteric heats of sorption (q_{st}) for FDHPs and FDLB calculated using the sorption data between 4 and 25 °C and between 25 and 37 °C

For FDHPs, q_{st} calculated using the data between 4 and 25 °C followed the similar trend to that of FDLB, although its value was a little lower than that of FDLB. However, q_{st} calculated using the data between 25 and 37 °C gave negative values, which might imply the endothermic interaction between the water molecules and the platelet membrane at higher temperature levels.

3.3 Moisture diffusion coefficient

The dynamic sorption data were fitted to Eq. (6) using the least square method to get the moisture diffusion coefficient. The calculated D_{eff} was 8.24×10^{-12} m^2/s with R^2=0.92 when the data within the initial 1600 s were used. The delays in mass gain were observed (Fig. 6), which was also illustrated in moisture sorption by polymer membranes (Marais *et al.*, 2000; Modesti *et al.*, 2004). This phenomenon may be caused by the time needed for the moisture content of the surface to reach equilibration with the environment.

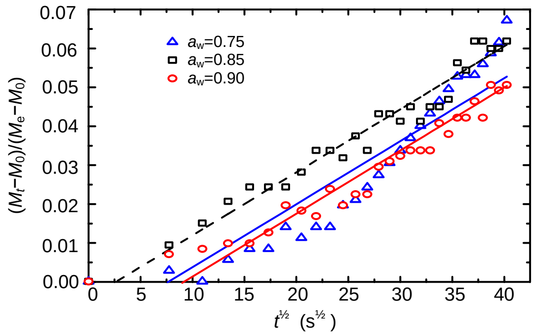

Fig. 6 Plots of relative mass gain as a function of the square root of time at three different water activities at 25 °C

The experimental data at the initial stage were used, because collapses of the freeze-dried samples were observed after 0.5 h or longer. The initial states of FDHPs and FDLB were porous, consisting of amorphous and crystal structures. After absorption of water, the fluidity of the structure would increase, which may be reflected by the change of glass transition temperature of the sample. According to Chen *et al.* (2000), if FDHPs and FDLB are viewed as the binary mixture of water and trehalose, the estimated glass transition temperature of the sample will drop below 25 °C when the moisture content (dry basis) is higher than 0.092.

The occurrence of collapse during the prehydration of freeze-dried sample would hinder this process. Considering the fact that trehalose tends to form a stable shell which may prevent further water vapor permeation, some inner parts of the sample may keep dry (Aldous *et al.*, 1995). Compared with thinner sample, thicker sample will face more difficulties brought by collapse. Using atmosphere with lower water activity for prehydration may help to solve this problem although longer time is needed.

4 Conclusions

Human platelets and its lyophilization buffer were freeze-dried. The moisture sorption isotherms were measured with gravimetric method at 4, 25, and

37 °C. The BET and GAB parameters were fitted and the net isosteric heat of sorption was deduced. The presence of platelets led to changes of the moisture sorption characteristics especially at 37 °C, for which the fundamental reason remains to be investigated. The sorption dynamics of freeze-dried platelets were also studied at room temperature 25 °C. Since the freeze-dried sample started to collapse after some time of prehydration, the moisture diffusion coefficient was obtained using the initial dynamic data. The knowledge obtained here are helpful for better design of the prehydration protocol for FDHPs.

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