A “Release” Protocol for Isothermal Titration Calorimetry

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ABSTRACT Isothermal titration calorimetry (ITC) has become a standard method for investigating the binding of ligands to receptor molecules or the partitioning of solutes between water and lipid vesicles. Accordingly, solutes are mixed with membranes (or ligands with receptors), and the subsequent heats of incorporation (or binding) are measured. In this paper we derive a general formula for modeling ITC titration heats in both binding and partitioning systems that allows for the modeling of the classic incorporation or binding protocols, as well as of new protocols assessing the release of solute from previously solute-loaded vesicles (or the dissociation of ligand/receptor complexes) upon dilution. One major advantage of a simultaneous application of the incorporation/binding and release protocols is that it allows for the determination of whether a ligand is able to access the vesicle interior within the time scale of the ITC experiment. This information cannot be obtained from a classical partitioning experiment, but it must be known to determine the partition coefficient (or binding constant and stochiometry) and the transfer enthalpy. The approach is presented using the partitioning of the nonionic detergent C12EO7 to palmitoyloleoylphosphatidylcholine vesicles. The release protocol could also be advantageous in the case of receptors that are more stable in the ligand-saturated rather than the ligand-depleted state.

INTRODUCTION

Motivation

In a recent review, White et al. (1998) stressed that partitioning studies of, e.g., peptides into lipid membranes suffer from the fact that “[u]nfortunately, there is no general way to establish with certainty the transbilayer distribution of peptides.” Seelig (1997) defined a correction factor γ to rescale the lipid or receptor concentration to the fraction that is accessible to the solute or ligand. However, apart from a few elegant approaches to determining γ for special systems (e.g., Wenk et al., 1997; Lin et al., 1994), the majority of papers thus far had to be based on reasonable assumptions regarding γ. It must be considered difficult and thus dangerous to make such an assumption, because a variety of effects and pathways have to be taken into account (see below).

Idea

Here we present a rather general approach to the transbilayer distribution problem. The basic idea is to compare a sample in which the solute was added “from outside” to a vesicle solution with another one, obtained by diluting a vesicle solution preloaded with solute in both the outer and inner monolayers. The partitioning data will agree in the case of fast membrane permeation of the solute (compared to the time scale of the experiment) but differ from each other if the ligand cannot cross the membrane, because then incorporation and release protocols lead to different kinetically entrapped nonequilibrium states (Fig. 1). Consequently, a consistent fit of incorporation as well as release data will be possible only in terms of the correct assumption regarding membrane permeability. This approach should be applicable to all kinds of partitioning experiments (cf. White et al., 1998)—those that are based on the macroscopic separation of the vesicles from the aqueous phase or part of it (equilibrium dialysis, centrifugation), as well as titration methods employing, for example, a spectroscopic parameter (CD, fluorescence). Furthermore, it applies to the case of partitioning of molecules into the membrane phase as well as to the specific binding of ligands to membrane receptors.

Isothermal titration calorimetry

In the past, ITC (Wiseman et al., 1989) has been established as an important method for the study of partitioning of solutes or surfactants into lipid membranes (Seelig, 1997; Heerklotz et al., 1996; Keller et al., 1997; Opatowski et al., 1997; Rowe et al., 1998; Wenk et al., 1997; Wenk and Seelig, 1998) and the binding of ligands to receptors reconstituted into lipid vesicles (Lin et al., 1994). We are aware of only two ITC studies that applied vesicles preloaded with solute, which could also serve to specify the transmembrane distribution of the additive (although this was not discussed in the original papers). Zhang and Rowe (1992) performed single injections of vesicles loaded with alcohols into alcohol solutions of different concentrations. Vanishing titration heats indicate that the free alcohol concentration in the syringe matches the known concentration in the cell. This method is very labor-intensive but has the advantage that no assumption regarding a constant partition coefficient has to be made. Opatowski et al. (1997) injected water into a
Then, about half of the lipid (protocols are reciprocal to each other for permeable membranes but give leased upon injection into buffer (release protocol the membrane, whereas solute preloaded into vesicle membranes is re- of injecting vesicles to free solute leads to a gradual uptake of solute into

Example for presentation

Assessing membrane permeability

Generally, reasonable estimates of \( \gamma \) are rather difficult to obtain, because a variety of possible permeation pathways with different kinetics have to be taken into account. In addition to the diffusive transport of solutes dissolving to a significant amount in the hydrophobic core of the membrane, small molecules may redistribute through small, transient membrane pores arising from density fluctuations in the bilayers (Jansen and Blume, 1995). The flip-flop rates of phospholipids in bilayer membranes devoid of proteins are slow compared to the timescale of ITC experiments (Yeagle, 1993). Lipids that readily undergo transbilayer diffusion must have a weakly polar headgroup (Zachovski, 1993). Some amphiphilic dye molecules can be induced to undergo flip-flop, but only in the presence of a transmembrane electrical potential (Melikyan et al., 1996). Fast membrane permeation has been reported for non-ionic detergents such as oligo (ethylene oxide) dodecyl ethers and octyl glucoside (Le Maire et al., 1987; Wenk et al., 1997). Molecules which can induce membrane leakage or pore formation can also access the inner monolayer, even if they have large polar groups. An example of this is pore formation by certain peptides which form amphipathic helices (Matsuzaki et al., 1997; Longo et al., 1998; Wenk and Seelig, 1998). Furthermore, an area expansion of the outer monolayer relative to the inner one by more than 5\%, which can be caused by nonsymmetrical incorporation of amphiphilic molecules, exerts a critical mechanical tension, giving rise to transient ruptures and, in turn, solute influx to the vesicle interior (Longo et al., 1998). Finally, solute permeation rates may substantially depend on the packing properties of the lipid membranes (Huster et al., 1997).

Experimental

The lipid palmitoyloleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Birmingham, AL), and the detergent hepta (ethylene oxide) dodecyl ether (C\(_{12}\)EO\(_{7}\)) was from Nikko Chemicals (Tokyo, Japan). The substances were used without further purification.

POPC was suspended in water by vortexing and subsequent extrusion through Nucleopore polycarbonate membranes of 100 nm pore size in a Liposofast minieextruder. This procedure was checked to yield essentially homogeneous unilamellar vesicles of 100 nm diameter and to cause no significant loss of material. The detergent was dispersed in water and vortexed rapidly.

The experiments were done using a Microcal MCS isothermal titration calorimeter (ITC) (Wiseman et al., 1989). As recommended by the manufacturer, a prior 1-\( \mu l \) injection was carried out without taking into account the corresponding observed heat, because the first injection is subject to somewhat larger errors. In the partitioning experiment, a 15 mM POPC vesicle suspension is titrated (14 injections of 3 \( \mu l \) each) into the calorimeter cell filled with a 50 \( \mu l \) detergent dispersion. For the release experiment, an appropriate amount of POPC is suspended in a 6.4 mM C\(_{12}\)EO\(_{7}\) dispersion to a final phospholipid concentration of 15 mM. The mixture is then vortexed and extruded as described above. This procedure ensures that the detergent is equally incorporated into both monolayers of the vesicle bilayer. The mixed suspension is placed in the syringe and injected into water in 14 aliquots of 3 \( \mu l \) each at 5-min intervals.

The fitting procedures were performed using MicroCal Origin with a user-defined script.
THEORY

General Eq.

In accord with the example presented here, we will denote the solute by D (detergent) and the lipid by L. We emphasize that the same derivation holds for the specific binding of ligands to receptors.

The system enthalpy $H$ can be written as the sum of the partial molar enthalpies $h$ of the components (indices: lipid L and detergent D) in the different environments (super-scripts: bilayers b and water w), weighted with the respective mole numbers $N$:

$$H = N_L \cdot h_L + N_D^b \cdot h_D^b + N_D^w \cdot h_D^w$$  \hspace{1cm} (1)

Note that lipid monomers can be neglected to a very good approximation, so that $N_L^b = N_L^w = N_L$. One can rewrite Eq. 1 using the molar concentrations of the detergent situated in bilayers and in water, $D_b$ and $D_w$, and the lipid concentration $L$, respectively, in the volume $V$:

$$H = V \cdot [L \cdot h_L + D_b \cdot h_D^b + D_w \cdot h_D^w]$$  \hspace{1cm} (2)

Let us assume $h_D^b$, $h_D^b$, and $h_D^w$ are constants (cf. Errors section):

$$h_D^b, h_D^b, h_D^w \equiv \text{const.}$$  \hspace{1cm} (3)

For the determination of the heat, $Q$, arising from the reequilibration after the initial mixing, we have to consider a closed system exchanging no material but only heat with the outside. Such a system must include the cell content, the syringe, and the access tube of the cell, to which some cell content is displaced because of the injection. Then $Q$ is given by the change in the enthalpy content of this system:

$$Q = \Delta H(\text{cell}) + \Delta H(\text{access tube}) + \Delta H(\text{syringe})$$  \hspace{1cm} (4)

For the protocols discussed below, the injection volumes are small compared with the cell volume, and the concentrations in the cell are low compared to those in the syringe. Then we may neglect the enthalpy content of the overflown sample:

$$\Delta H(\text{access tube}) \equiv 0$$  \hspace{1cm} (5)

An equation for the heat $Q$ is derived in the Appendix. The observed heat, $q_{\text{obs}}$, is normalized with respect to the total injected mole numbers $\Delta N_L$ and $\Delta N_D$:

$$q_{\text{obs}} = \frac{Q}{\Delta N_L + \Delta N_D^b} = \frac{Q}{V \cdot (\Delta L + \Delta D_b)} \equiv \frac{Q}{\Delta V \cdot (L^\text{SV} + D_b^\text{SV})}$$  \hspace{1cm} (6)

Note that the injected numbers of moles are related to the volume of the injection, $\Delta V$, and the total lipid and detergent concentration in the syringe ($L^\text{SV}$ and $D_b^\text{SV}$) or to the concentration changes of the lipid and detergent, ($\Delta L$ and $\Delta D_b$) in the sample volume, $V$. Applying Eq. 6 to Eq. 24 derived in the Appendix, we obtain

$$q_{\text{obs}} = \Delta h_{b}^{w-b} \cdot \left[ X^{\text{SV}} \cdot \frac{\partial D_b}{\partial D_t} + (1 - X^{\text{SV}}) \cdot \frac{\partial D_b}{\partial L} \right] \cdot \frac{D_b^\text{SV}}{D_b^\text{SV} + L^\text{SV}}$$

$$+ q_{\text{dil}}$$  \hspace{1cm} (7)

where $X^{\text{SV}}$ denotes the total detergent mole fraction in the syringe, and the molar detergent transfer heat from water to bilayers is $\Delta h_{b}^{w-b} = h_{b}^{w} - h_{b}^{b}$. The constant $D_b^\text{SV}/(D_b^\text{SV} + L^\text{SV})$ considers the degree of binding of the solute before the injection. The term $q_{\text{dil}}$ includes the molar heat of dilution of the injectant, which can be assumed to be constant and is measured separately by a blank experiment in most cases.

The classic “incorporation” protocol: injecting vesicles to a solute dispersion

If the syringe contains a suspension of pure lipid vesicles ($X^{\text{SV}} = 0, D_b^\text{SV} = 0$), Eq. 7 simplifies to the known function for the classic incorporation protocol (Heerklotz et al., 1996; Keller et al., 1997; equivalently to Seelig and Ganz, 1991; Seelig, 1997):

$$q_{\text{obs}} = \Delta h_{b}^{w-b} \cdot \frac{\partial D_b}{\partial L} + q_{\text{dil}}$$  \hspace{1cm} (8)

The partial derivative $\partial D/b / \partial L$ takes account of the detergent transfer from the water to the membrane during reequilibration after an injection. For the partition coefficient $P$ defined in terms of mole fractions, we find (Tanford, 1981)

$$P = \frac{D_b \cdot W}{(D_b + L) \cdot (D_b - D_t)}$$  \hspace{1cm} (9)

with $W \approx 55.5$ M. Note that alternative definitions (cf. White et al., 1998; Seelig, 1997; Lasch, 1995) can be treated analogously. Solving Eq. 9 for the concentration of membrane-bound detergent, $D_b$, one obtains (Heerklotz et al., 1996; Keller et al., 1997)

$$D_b = \frac{1}{2 \cdot P} \cdot \left[ P \cdot (D_t - L) - W \right]$$

$$+ \sqrt{P^2 \cdot (D_t + L)^2 + 2 \cdot P \cdot W \cdot (L - D_t) + W^2}$$  \hspace{1cm} (10)

and, subsequently, the partial derivative:

$$\frac{\Delta D_b}{\Delta L} = - \frac{1}{2}$$

$$\frac{1}{2} \cdot \sqrt{P^2 \cdot (D_t + L)^2 + 2 \cdot P \cdot W \cdot (L - D_t) + W^2}$$  \hspace{1cm} (11)

The $q_{\text{obs}}$ are plotted versus the average $L$ corresponding to the respective injections. The dilution heat, $q_{\text{dil}}$, is estimated by injecting the same vesicle suspension into buffer (or water). The total detergent concentration, $D_b$, remains essentially constant during the experiment because the syringe
volume is small compared to the cell volume. Hence, Eqs. 8 and 11 constitute a fitting model that allows the determination of $\Delta h_b^{\text{obs}}$ and $P$.

### The release experiment: injecting a mixture to water

Let us consider an experiment in which the syringe is filled with a mixture of lipid vesicles and solute prepared in a way that the solute is evenly distributed inside and outside the vesicles. The syringe content can be specified in terms of the molar lipid concentration $L^{\text{sv}}$ and the total detergent mole fraction $X^{\text{sv}}$. The cell is filled with buffer (or water).

Again, the observed normalized heats $q_{\text{obs}}$ are plotted versus the average lipid concentration in the cell, $L$. However, in this case, the average total detergent concentration $D_t$ also varies. Generally, it can be calculated from known quantities using

$$D_t = \frac{X^{\text{sv}}}{1 - X^{\text{sv}}} \cdot L + D_t^0$$  \hfill (12)

with the initial detergent concentration in the cell, $D_t^0$, and the detergent injected into the cell from the syringe, which obeys a fixed ratio $X^{\text{sv}}/(1 - X^{\text{sv}})$ to the injected lipid (abscissa $L$). Note that $D_t^0 = 0$ for the release experiment and $X^{\text{sv}} = 0$ for the incorporation protocol. For the fitting procedure we need Eqs. 7 and 10 and the derivative of Eq. 10 with respect to the total detergent concentration, $D_t$:

$$\frac{\partial D_t}{\partial L} = \frac{b}{2} - \frac{K \cdot b \cdot (L \cdot b - D_t) + b}{2 \cdot \sqrt{K^2 \cdot (L \cdot b - D_t)^2 + 2 \cdot K \cdot (L \cdot b + D_t) + 1}}$$ \hfill (16)

and

$$\frac{\partial D_t}{\partial b} = \frac{1}{2} - \frac{K \cdot (D_t - L \cdot b) + 1}{2 \cdot \sqrt{K^2 \cdot (L \cdot b - D_t)^2 + 2 \cdot K \cdot (L \cdot b + D_t) + 1}}$$ \hfill (17)

### Accessibility coefficients considering membrane impermeability

To consider the fact that not all of the molecules are able to redistribute across the bilayer, we have to replace the total lipid and detergent concentrations by effective concentrations that do not include molecules trapped inside the vesicles, substituting

$$D_t \rightarrow \gamma_t \cdot D_t$$ \hfill (18)

$$L \rightarrow \gamma_L \cdot L$$ \hfill (19)

At this point, the principal difference between the two protocols becomes obvious (cf. Fig. 1). Whereas upon lipid vesicle titration to a solute solution (incorporation protocol) all of the solute is free to distribute ($\gamma_0 = 1$, $\gamma_L \approx 0.5$ for LUV; cf. Fig. 1, middle row, right), both lipid and solute may be partially trapped in the case of the release experiment, injecting the mixture to water ($\gamma_0 = \gamma_L \approx 0.5$; cf. Fig. 1, middle row, left). Because of the substitutions in Eqs. 18 and 19, some of the previously introduced equations (e.g., 10, 11, 13, 16, 17) become functions of $\gamma_0$ and $\gamma_L$, and thus, the results of the fit procedure depend on the assumption of whether the membranes are permeable for the ligand.

### RESULTS AND DISCUSSION

#### Example C_{12}EO_7/POPC

Fig. 2 shows the data measured by means of the incorporation protocol (circles) and of the release protocol (squares, corresponding to two attempts). The experimental parameters are displayed in Table 1.

A direct fit of $\gamma_L$ and $\gamma_0$ to the experimental data of both incorporation and release protocols is theoretically possible but technically somewhat difficult, because both data sets are described by the same function but with different values for $\gamma_0$. Instead, we are going to show that a consistent evaluation of all data is only possible based on the correct assumption regarding the membrane permeability. We performed separate as well as a simultaneous fitting evaluation of the data sets according to Eq. 7 with Eqs. 10, 11, 13, 18, and 19.

The incorporation data alone can be modeled quite well in terms of the parameter sets, assuming both permeable or impermeable membranes (cf. Table 1, experiment I). The two fits correspond to essentially the same curve (not
The curves shown in Fig. 2 correspond to a simultaneous fit of the incorporation as well as the release data. Assuming impermeable membranes, no parameter set \((P, \Delta h_{D}^{\text{w-b}})\) was found to describe the data (best fit displayed by dotted lines). In contrast, with \(\gamma_{L} = \gamma_{D} = 1\), a good consistent fit was possible (solid lines in Fig. 1, parameters in Table 1: \(I + R\)).

The last column of Table 1 (I2) refers to a partitioning experiment performed at a considerably higher detergent concentration. It should be noted that the lipid of the first injections of the I2 setup is solubilized to micelles (Heer-klotz et al., 1996; Wenk and Seelig, 1997). The data displayed in Table 1 refer to later injections, when the sample has completely reconstituted to bilayers. Note that the parameters obtained for I2 differ significantly from those for I and R. This indicates that the membrane compositions that are present during both the I and R experiments (cf. Table 1, row \(X_{L}\)) should match essentially to minimize errors due to nonideal mixing effects (cf. below).

### Applications of the release protocol

The release protocol introduced here must be considered an interesting alternative or supplement to the classic ITC protocols. Here we have shown that the application of both the classic partition and the release protocols serves to solve the membrane redistribution problem. However, there are cases where the application of the release protocol should be superior, even though the transbilayer distribution of the ligand is not an issue. For example, proteins that are destabilized upon extraction of the ligand can be handled in the ligand-saturated state until the experiment starts. The partial release of the ligand during the experiment is restricted to a minimum time and subject to constant conditions such as temperature, chemical interactions, mechanical agitation, and so on. The range of binding constants measurable by the release protocol is similar to that of the classic binding or partitioning protocols (up to, e.g., \(10^9/\text{M}\)). We note that the resolution for the different parameters, particularly for the three-parameter fit required for specific binding, can be considerably improved by a simultaneous fit of two data sets measured with different solute/ligand contents in the titrant.

To discuss the limitations of the transbilayer distribution problem, it seems noteworthy that complete redistribution or the absence of redistribution constitute the limiting cases.
There may be more complicated systems with a partial permeation of the solute. First, the term permeability refers to a distinct time scale. In the context of ITC, permeable means that equilibrium is reached within the recording time after each injection, typically in the range of 5–30 min. Impermeable means that essentially no membrane permeation has occurred within the time required for the titration, e.g., 1–5 hours. Intermediate redistribution rates will result in intermediate and variable values for γ. Second, a permeability threshold might exist, making the membranes permeable beyond a distinct solute content. Then the state of the system depends not only on its composition but also on its history. In these intermediate cases, ITC experiments may not be applicable. Whereas the application of the classic partition experiment alone may yield a false result, the additional application of the release protocol should indicate the failure by allowing no consistent fit, whatever value (constant) of γ is assumed.

**Errors**

To address possible error sources, we have simulated data sets under varying conditions and assumptions in a spreadsheet and subsequently evaluated these data using the fit procedures explained above. The simplification of neglecting the sample replacement due to the injections into a calorimeter of fixed cell volume is justified for the examples presented here. For larger syringes (i.e., 250-μl syringe and 1300-μl cell) it can cause a significant error. The assumption of P and Δh°Dw-b being constant is a rather poor approximation. Indeed, the partition coefficients of C_{12}EO_{7} (Heerklotz et al., 1994) and other detergents (Paternostre et al., 1995; Lasch, 1995; Keller et al., 1997) decrease with increasing detergent content in the membrane, and the heats of binding may also depend on membrane composition (E惋nd and E惋nd, 1994; Heerklotz et al., 1998). Then the fit parameters reflect an average value with some preference for the conditions present upon the first injections, where the highest heats are measured. This effect could account for the partition coefficients to decrease somewhat from the R to the I experiment and further to J2 (cf. Table 1), because the detergent contents in the membrane X_{i} corresponding to the beginning of the titration increase in this order.

Note that the correct separation between the different parameters is achieved by means of the model and must be affected in the case of wrong model assumptions. This is illustrated, e.g., by the different transfer heats obtained assuming permeable or impermeable membranes, although, physically, only the partitioning of the detergent depends on the membrane permeability (cf. Table 1). This behavior also gives rise to some deviations in the partition coefficients obtained in systems with varying transfer heat and vice versa. In our simulations of data for partition coefficients or transfer heats varying by ~30% during the titration, the reproduction of the other, constant value failed by up to 20%, which can be considered satisfactory. Thus the composition dependence of the transfer heat in the system presented here (cf. Heerklotz et al., 1997) may account, to some extent, for the fact that the partition coefficients obtained by ITC are somewhat lower than previously published values of about (4–5) · 10^{5} (Heerklotz et al., 1994).

We summarize that the slight systematic deviations of the fit curves from the data do not justify the introduction of additional adjustable parameters describing the composition dependence of the partition coefficient P (or, equivalently, of Δh°Dw-b) (Heerklotz et al., 1996; Keller et al., 1997).

Another error source can be the heat of injectant dilution for the release protocol, which cannot directly be measured and could differ somewhat from the value measured for pure lipid vesicles. To address this problem, we repeated the fit procedures, leaving γ_{bil} for the release experiment as a third adjustable parameter. However, this did not significantly affect the results.

We note that the assumption of γ_{D} = 0.5 for impermeable LUV does not take account of the aqueous solute concentration in the syringe in the frame of the release experiment. In the general case one has to use the following relation to estimate γ_{D} for impermeable LUV:

\[
γ_D = D_0^{sy} \cdot 0.5 + (D_0^{sy} - D_{0t}^{sy}) \cdot \left(1 - \frac{V_{VES}}{V_t}\right) \tag{20}
\]

For 15 mM (spherical) LUV of POPC with 100 nm diameter, the vesicles enclose a volume fraction of V_{VES}/V_{t} = 5 vol%. For the example presented here, D_{0t}^{sy} ≈ D_{0t}^{sy}, and the second term in Eq. 20 vanishes. For low lipid concentrations or partition coefficients, it could be appropriate to correct γ_{D} using Eq. 20.

**Conclusions**

We derived a general formula (Eq. 7) that serves to model all possible titration calorimetry protocols assessing the nonsaturating partitioning of a solute between water and lipid vesicles, as well as the specific binding of a ligand to a receptor.

We applied this equation to model the data of a release protocol based on the injection of solute-loaded vesicles into buffer.

Generally, the question of whether a molecule permeates the membrane and, thus, reaches the inner lipid monolayer or receptors exposed there must be answered for a proper evaluation of binding data.

Taking into account the data obtained by means of the new release protocol as well as those from the classical incorporation/binding protocol, one can clearly distinguish whether the solute/ligand penetrates the bilayer and detect the case where ITC fails to establish the partitioning behavior because a partial redistribution takes place in the experimental time scale.

The approach was presented for the example of the detergent C_{12}EO_{7}, which is known to quickly penetrate lipid bilayers. This fact could be confirmed successfully.
The same principal approach is also applicable to non-calorimetric partitioning and binding assays, such as step-wise and continuous titrations into a fluorescence spectrometer, and others.

The calorimetric release protocol can also be expected to be advantageous, apart from the membrane permeability problem. For example, proteins, being more stable in the presence of ligand, favor the release protocol, reducing the time and treatment in the ligand-depleted state to a minimum.

**APPENDIX: DERIVATION OF EQ. (7)**

To apply Eq. 4 we have to determine the enthalpy variation of the syringe and cell contents upon an injection. The syringe concentrations \(L^{sy} \), \(D_w^{sy} \), and \(D_b^{sy} \) are constant (and so are the molar enthalpies), and the only change in the syringe content is the volume diminishing by \(DV \), yielding with Eq. 2

\[
\Delta H(\text{syringe}) = -\Delta V \cdot (L^{sy} \cdot h_b^b + D_w^{sy} \cdot h_b^b + D_b^{sy} \cdot h_b^b)
\]  

(21)

For the cell content, we assumed the partial molar enthalpies to be constant (Eq. 3), but the total concentrations as well as the distribution of the detergent between water and membrane may vary. The cell volume is constant \(V_o \). Hence, we have to consider the total differential:

\[
\Delta H(\text{cell}) = V_o \cdot \left[\left(\frac{\partial D_b}{\partial L} \cdot \Delta L + \frac{\partial D_b}{\partial D_t} \cdot \Delta D_t\right) \cdot h_b^b + \left(\frac{\partial D_w}{\partial L} \cdot \Delta L\right) \cdot h_b^w\right]
\]  

(22)

Trivially, \(\Delta L / \Delta L = 1\) and \(\Delta L / \Delta D_t = 0\). With the mass balance inside the cell, \(D_t = D_b + D_w\), we find that \(\Delta D_b / \Delta D_t = 1 - (\partial D_b / \partial D_t)\) and \(\Delta D_w / \Delta D_t = -(\partial D_w / \partial D_t)\). The mass balance between the syringe and the cell yields (with the approximation, Eq. 5) the relations \(\Delta V \cdot L^{sy} = V_o \cdot \Delta L\) and \(\Delta V \cdot (D_w^{sy} + D_b^{sy}) = V_o \cdot \Delta D_t\). Considering this information, Eq. 22 becomes

\[
\Delta H(\text{cell}) = \left[h_b^b - h_b^w\right] \cdot \left[V_o \cdot \Delta D_t + \frac{\partial D_b}{\partial L} \cdot V_o \cdot \Delta L\right] + V_o \cdot \Delta L \cdot h_b^w + \Delta V \cdot (D_w^{sy} + D_b^{sy}) \cdot h_b^w
\]  

(23)

Inserting Eqs. 21, 23, and 5 into 4, we find the heat consumed or released upon the injection, \(Q\):

\[
Q = \left[h_b^b - h_b^w\right] \cdot \left[\frac{\partial D_b}{\partial D_t} \cdot V_o \cdot \Delta D_t + \frac{\partial D_b}{\partial L} \cdot V_o \cdot \Delta L + \Delta V \cdot (D_w^{sy} + D_b^{sy})\right]
\]  

(24)

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