A more efficient device for preparing model-membrane liposomes by the rapid solvent exchange method

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We modified the original design for a rapid solvent exchange (RSE) device with the intent of making the RSE method (i) more efficient and (ii) easier to adopt and implement. Our modifications improved solvent-removal kinetics by a factor of 2, while reducing sample-prep time by a factor of 3. In this paper, we develop the kinetic model that informed the device revision and we address several RSE parameters that have not yet been discussed in the literature. We also provide detailed mechanical drawings and present solvent-removal efficiency data that confirm the improved performance of our device.


I. INTRODUCTION

Some years ago, we published a paper describing the so-called “rapid solvent exchange” (RSE) method for preparing model-membrane liposome suspensions.1 The RSE method is specifically designed to preserve compositional homogeneity in membrane mixtures by avoiding intermediary solid states during the sample preparation process. This feature sets it apart from the two most commonly used liposome-prep techniques: film deposition and lyophilization.

The focus of our original paper was not device design or process optimization. Rather, our aims were to explain the reasoning that led us to develop RSE, to characterize the physical properties of RSE liposomes, and to present data supporting our contention that artifactual demixing should be a matter of concern for anyone using conventional methods of sample preparation. At the time, our research centered on membrane mixtures in the regime of high-cholesterol content, so we were primarily concerned with maintaining homogeneity under those conditions.

We have recently come to believe that the utility and importance of the RSE method extends well beyond the range of high-cholesterol content,2 and for this reason we have undertaken to revisit and improve the design of our original RSE device. Our intent has been to make RSE more efficient (in terms of both solvent-removal kinetics and sample-prep time) and easier to implement (i.e., for laboratories that are currently using film deposition or lyophilization). As a result of our redesign, we improved solvent-removal efficiency by a factor of 2 and reduced sample-prep time by a factor of 3.

In this paper, we present a kinetic model used to guide the optimization of RSE solvent-removal efficiency. We address various design parameters that were not discussed in the original paper, and we present experimental solvent-removal data that confirm both the predictions of the model and the improved performance of our revised device. Finally, we provide via EPAPS detailed information (e.g., mechanical drawings for custom-fabricated parts) that will enable any interested research group to assemble its own RSE device.

II. MODEL FOR RESIDUAL SOLVENT REMOVAL

In order to approach systematically the improvement of our device, we begin with a quantitative description of the RSE process itself. The rate-limiting stage of solvent removal is not the removal of bulk solvent—which boils away at reduced pressure on a timescale of ~1 s—but rather involves the more gradual removal of residual solvent, which is removed on a timescale of ~10–100 s.1 For this process, a simple two-step kinetic model (Fig. 1) serves to describe residual-solvent removal, in which A represents solvent dissolved within the aqueous phase, V represents headspace solvent vapor (in exchange with the aqueous phase), and T represents trapped solvent (i.e., removed from the sample tube and irreversibly captured by the vacuum trap). The rate coefficients associated with each transfer step depend, of course, on a variety of variables, some of which will be discussed under Sec. III.

Figure 1 leads to a relatively simple time-dependent description of residual solvent removal. Starting with the kinetic model, a second-order linear homogenous ODE can be derived that relates the three rate coefficients to A and its rates of change:

\[ d^2A/dt^2 + (k_1 + k_{-1} + k_2) dA/dt + k_1k_2A = 0. \]  

Equation (1) then implies a biexponential solution in terms of the rate coefficients and initial solvent residue concentration, \( A_0 \):

\[ A(t) = \frac{A_0}{\lambda_2 - \lambda_1} \left[ (\lambda_2 - k_1)e^{-\lambda_1 t} - (\lambda_1 - k_1)e^{-\lambda_2 t} \right], \]  

where \( \lambda_1 = (p-q)/2 \), \( \lambda_2 = (p+q)/2 \), with \( p = k_1 + k_{-1} + k_2 \) and \( q = \sqrt{p^2 - 4k_2k_1} \). This expression describing the time-dependent removal of residual solvent serves as a basis from
which to approach systematically the optimization of the solvent-removal process, as discussed below under Sec. IV.

III. DEVICE DESIGN

A. Precombination of phases

The first improvement to our original RSE device design has nothing to do with solvent-removal kinetics. Rather, in the interest of sample-throughput efficiency, we removed the injection assembly entirely, opting simply to precombine the organic and aqueous phases instead of spraying the organic solution against vortexing aqueous buffer under vacuum (as was done in the original RSE procedure).1

The spray-injection step added considerable overhead to RSE sample-prep times, since the injection needle needed to be cleaned by back flushing after every sample. When we originally began preparing samples by RSE, we felt that the most conservative approach would be to avoid prevaporization contact between the organic and aqueous phases,5 so this was the rationale for incorporating an injection assembly in our original device design.

Nevertheless, subsequent experience failed to produce evidence of any artifacts associated with precombining the phases, so we abandoned injection altogether, replacing it with precombination as our best-practice procedure. We no longer worry that precombination could compromise the integrity of the RSE method, and it does speed up sample preparation by about a factor of 3.

B. \(k_1\) and \(k_{-1}\)

In order to optimize solvent removal kinetics, let us now consider the coefficients \(k_1\) and \(k_{-1}\), which describe A \(\rightarrow\) V exchange. These coefficients scale in proportion to the interfacial area between the A and V phases, and so maximizing this area is clearly important. Rather than employing a special chamber to serve this purpose, our RSE device simply vortexes each sample within its own tube, forming the aqueous buffer into a rapidly spinning, thin cylindrical shell (Fig. 1). This simple and effective strategy avoids additional transfer steps through use of a piece of equipment (vortexer) found in every laboratory that prepares liposomes. By employing different sample-tube adapters (see EPAPS archive of mechanical drawings), our current device can accept sample tubes ranging from 3 to 100 cm³ in capacity, accommodating a wide range of sample volumes.

The effective exchange area of such a turbulent, vortexing liquid is, of course, hard to define,6 and so it is hard to say a priori by exactly what factor the rate constants are increased for any particular set of experimental conditions. However, as a first approximation, it is reasonable to suppose that both \(k_1\) and \(k_{-1}\) will be increased by about the same factor, so the vortexing action can be viewed as essentially driving the A \(\rightarrow\) V step toward equilibrium, leaving \(k_2\)-dependent V \(\rightarrow\) T transfer to serve as the rate-limiting step for residual solvent removal.

C. \(k_2\) and \(f_{\text{gas}}\)

The magnitude of the trap-transfer coefficient, \(k_2\), should depend on a number of variables, including tube-neck diameter, the pressure gradient along the tube-to-trap path (\(\nabla P\)), and the vapor-phase solvent diffusion constant. Of these variables, \(\nabla P\) is most easily manipulated, and this is the one we exploit to increase RSE efficiency.

Our strategy for manipulating \(\nabla P\) is to introduce a flow-controlled source of inert gas, \(f_{\text{gas}}\), at the bottom of the vortexing sample tube. This flushing gas constantly sweeps across the surface of the vortexing liquid, helping to carry solvent vapor toward the trap and thereby increasing \(k_2\).

D. Assumptions

For the purpose of process analysis and optimization, we shall hereafter treat \(k_2\) as a function of \(f_{\text{gas}}\), while assuming that \(k_1\) and \(k_{-1}\) remain constant. Of course, these latter rate coefficients cannot be perfectly independent of \(f_{\text{gas}}\), but as the solvents of interest for RSE are all volatile (e.g., chloroform) and mass-transfer rates of volatile compounds are widely agreed to be dominated by liquid-phase transport,7 it is certainly reasonable to treat \(k_1\) as independent of \(f_{\text{gas}}\). And since treating \(k_{-1}\) as constant may serve only to underestimate overall RSE efficiency, we feel confident that this approach is sound for our purpose.

IV. ANALYSIS AND OPTIMIZATION

Equation (2) implies two phases of residual aqueous solvent removal, a slow phase and a fast phase. Because we are interested in optimizing RSE efficiency, our attention will focus on the slow phase. However, we ought to first estimate
the timescale of the fast phase; and so we must obtain at least crude (i.e., one sig-fig), lower-limit estimates of all three rate constants, $k_1$, $k_{-1}$, and $k_2$.

A. Estimating coefficients

In order to obtain an estimate for $k_1$, we exploit the fact that $k_1$ corresponds to the mass transfer coefficient for solvent volatilization—a subject to which chemical and environmental engineers devoted much research over the last few decades. These studies found that volatilization rates depend on many fluid-dynamic variables, so that generally, in order to get a reliable estimate of $k_1$, a direct measurement must be made for each particular set of experimental conditions. However, another important finding reported in the same body of literature is the fact that $k_1$ values for high-volatility compounds can be inferred by simple proportionality from the reaeration rate, $k_{\text{ox}}$, measured for the same aqueous sample (e.g., for chloroform $k_1 = k_{\text{ox}}/2$). Therefore, in order to determine a crude $k_1$ for our RSE device, we simply estimate $k_{\text{ox}} \approx 1$ s$^{-1}$ for our rapidly spinning, thin cylindrical shell of aqueous buffer (see Sec. VI) and conclude that $k_1 = k_{\text{ox}}/2 \approx 0.5$ s$^{-1}$.

With a $k_1$ estimate in hand, it is a simple matter to infer an approximate value for $k_{-1}$ given the experimentally determined partition coefficient, $\alpha = k_{-1}/k_1$, for CHCl$_3$ between water and air. At 20 °C and 1 atm, $\alpha \approx 8$, implying $k_{-1} \approx 4$ s$^{-1}$ for our device.

Because the trap-transfer rates of solvent vapor and water vapor are both proportional to $k_2$, it is also straightforward to estimate $k_2$ by measuring the transfer rate for water and dividing this by its steady-state vapor pressure. Since we are seeking here a lower-limit estimate for $k_2$, we may simply use the equilibrium vapor pressure for water,

$$k_{2\text{H}_2\text{O}} = d(\text{H}_2\text{O})/dt$$

where $n_{\text{H}_2\text{O}} = \frac{P_{\text{eq}}V_{\text{ube}}}{RT} \approx 8.8 \times 10^{-6}$ mol, which, given a measured trap-transfer rate of 1.0 $\times 10^{-3}$ mol/s in the absence of flushing gas flow, implies that $k_2 \approx 1$ s$^{-1}$ for $f_{\text{gas}} = 0$ cc/s.

B. Solvent-removal efficiency and $\beta$

Taken together, these lower-limit estimates for $k_1$, $k_{-1}$, and $k_2$ suggest an upper-limit estimate of $\lambda_1^{-1} = 0.1$ s for the fast-phase timescale. As long as we restrict our observations to longer postvaporization times, we may therefore approximate the time-dependent description of residual solvent as

$$A(t) \approx \frac{\lambda_2 - k_1}{\lambda_2 - \lambda_1} A_0 e^{-\lambda_1 t}$$

and experimental solvent-residue time-course data can be expected to fit well a single-exponential with time constant $\lambda_1$.

With this simpler expression serving to describe the slow-phase removal of residual solvent, it is appropriate now to consider how $\lambda_1$ depends on $k_2$, the rate coefficient we can control using $f_{\text{gas}}$.

$$\lambda_1 = \frac{k_1 + k_{-1} + k_2 - \sqrt{(k_1 + k_{-1} + k_2)^2 - 4k_1k_2}}{2}$$

In order to predict the behavior of $\lambda_1$ as $k_2 > k_1$, $k_{-1}$ we can express the discriminant above in terms of $k_1/k_2$, $k_{-1}/k_2$ and discard terms of inverse second-order $k_2$ dependence, since they approach zero most rapidly in this limit. If we then invoke the binomial expansion and consider the leading term, we conclude that $\lambda_1 \rightarrow k_1$ as $k_2 > k_1$, $k_{-1}$. By similar reasoning, we see that $\lambda_1 \rightarrow k_2$ in the opposite limit, and we are left with the following observations:

$$\lambda_1 = k_2 \text{ as } k_2 \rightarrow 0$$

$$\lambda_1 = k_1 \text{ as } k_2 > k_1, k_{-1}$$

Of course, that the process-limiting time constant should behave in this way is perfectly clear from Fig. 1, but the analysis in terms of Eqs. (3) and (4) offers further insight when we ask exactly how solvent removal efficiency (i.e., $dA/dt$ or $d \ln A/dt$) depends on the parameter under our control: $k_2(f_{\text{gas}})$. And in order to produce a general expression, we can now appreciate the utility of expressing our answer in natural units of $k_1$, the rate-limiting coefficient for the RSE process:

$$\frac{(d \ln A/dt)}{k_1} = -\frac{\lambda_1}{k_1} = \frac{1 + \alpha + \beta - \sqrt{1 + \alpha^2 + \beta^2 + 2(\alpha + \alpha \beta - \beta)}}{2}$$

Here $\alpha = k_{-1}/k_1$, the solvent partition coefficient, of course. But we have also defined an independent variable $\beta = k_2/k_1 = \beta(f_{\text{gas}})$, which represents a gas-flow “knob” with which we can regulate solvent-removal efficiency. Since $\alpha$ can be treated as a constant, we can plot $(d \ln A/dt)/k_1$ as a function of $\beta$ only.

Figure 2 shows a plot of Eq. (6) parametrized with $\alpha = 8.2$. With reference to this figure, three clear predictions can now be made. First, for our device, $\beta = 1/0.5 = 2$ at $f_{\text{gas}} = 0$ cc/s, so we should expect the RSE process to

![Color online] Predicted dependence of chloroform-removal efficiency on the trap-transfer coefficient, $k_2$, for the RSE device described in this work. Both axes are expressed in natural units of $k_1$, and inset A indicates the efficiencies expected at $\beta = 2$ and 250, respectively.
achieve only about 20% of maximum (i.e., \( k_1\)-limited) efficiency in the absence of any flushing gas flow [Fig. 2 inset (a)]. Second, since both \( k_1 \) and \( k_{-1} \) are of order 1 for our device, whenever \( \beta > 100 \) we should expect the process-limiting time constant to approach \( k_1 \), since this is indeed the regime where \( k_2 \gg k_1, k_{-1} \) [Eq. (5)]. And third, if we increase \( f_{\text{gas}} \) to a point at which \( \beta = k_2 / k_1 \gg 250 \), then we can expect to achieve better than 97% of maximum device efficiency [Fig. 2 inset (b)] so that further increases to \( \beta \) should not appreciably improve performance.

V. EXPERIMENTAL VALIDATION

All these predictions are confirmed by the solvent-removal time course data shown in Fig. 3. In the upper panel, data are shown for \( \beta = 2 \) \(( f_{\text{gas}} = 0 \text{ cc/s})\), and from the slope we surmise that \( \lambda_1 = 0.092 \text{ s}^{-1} \) under these conditions. This is, as expected, about 20% of the efficiency \((\lambda_1 \approx 0.44 \text{ s}^{-1})\) implied by the middle panel, where \( f_{\text{gas}} \) has been increased to 1 cc/s [i.e., 60 SCCM (SCCM denotes standard cubic centimeter per minute at STP)] so that \( \beta = 280 \) (Table I), achieving twice the solvent-removal efficiency of the original RSE device.\(^{12}\) In the lower panel, \( f_{\text{gas}} \) is doubled to 2 cc/s \((\beta \approx 430)\), but \( \lambda_1 \) remains essentially unchanged, confirming that further increases in \( f_{\text{gas}} \) cannot appreciably improve solvent-removal efficiency. And finally, there is clearly agreement between the \( k_1 \) estimates derived from solvent-removal experiments (Fig. 3: \( \lambda_1 = 0.44 \text{ s}^{-1} = k_1 \) for \( \beta > 100 \) regime) and the completely independent, one-sig-fig, \( k_{\text{ox}} \)-based estimate we obtained from fluorescence quenching \((k_1 = 0.5 \text{ s}^{-1})\).

VI. MATERIALS AND METHODS

Palmitoyloleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids and purity was confirmed by thin layer chromatography on washed, activated silica gel plates.\(^{13}\) Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer and disodium ethylenediaminetetraacetic acid (EDTA) were from Fluka Chemie AG. Aqueous buffer \((2.5 \text{ mM PIPES pH} 7.0, 250 \text{ mM KCl, 1 mM EDTA})\) was prepared from 18 MΩ water (Barnstead E-Pure) and filtered through a 0.2 \( \mu \text{m} \) filter before use.

Each POPC liposome sample \((4.5 \times 10^{-7} \text{ mole total lipid per tube})\) was prepared by precombining 75 \( \mu \text{l} \) of a 6.9 mM chloroform-based POPC solution with 1.2 ml of aqueous buffer in a 13 \times 100 mm screw cap test tube. The tube was mounted on a laboratory vortexer and coupled to the sample manifold of our RSE device (see Ref. 3). The vortexer was actuated, the flushing-argon flow rate \((f_{\text{gas}})\) was confirmed, and then the manifold valve was quickly opened to a trap-protected vacuum system preset at \( \sim 25 \text{ torr} \). Vaporization of bulk chloroform is essentially instantaneous under these conditions, so postvaporization time points were clocked from the moment the manifold valve was opened. After the appropriate time elapsed, the vortexer was stopped, the manifold was vented, and the sample tube was removed from the device and sealed securely with a Teflon lined, gastight screw cap. For each time point, 20 replicate samples were pooled, extracted into deuterated benzene, and analyzed for chloroform residue by 1H-NMR as previously described.\(^{1}\)

For the specific fluid-dynamic conditions under which we prepared our liposomes (1.2 ml aqueous buffer vortexing in a 13 \times 100 mm tube attached to our sample manifold), we estimated the reaeration rate constant, \( k_{\text{ox}} \), by adding a water-soluble fluorescent dye (carboxyfluorescein), thoroughly degassing the fluorescent solution at 25 torr, and then venting the sample to atmosphere while recording the time course of fluorescence intensity reduction caused by the restoration of collisional oxygen quenching.

<table>
<thead>
<tr>
<th>( f_{\text{gas}} ) (cc/s)</th>
<th>Observed trap-transfer rate, ( d(H_2O)/dt ) (mol/s)</th>
<th>Implied ( k_2 ) (s(^{-1}))</th>
<th>Implied ( \beta )</th>
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</thead>
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<tr>
<td>0</td>
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<td>2</td>
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<td>280</td>
</tr>
<tr>
<td>2</td>
<td>1.9 \times 10^{-3}</td>
<td>215</td>
<td>430</td>
</tr>
</tbody>
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TABLE I. Dependence of \( k_2 \) on inert gas flow.
VII. CONCLUSIONS

We presented a detailed description of a more efficient device for preparing model-membrane liposomes by the RSE method. This improved design reduces sample-prep times by threefold and increases solvent-removal efficiency twofold. It is our hope that the information provided here and in the EPAPS archive will help our colleagues in model-membrane and liposome research to more easily adopt the RSE method in their own laboratories.

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3 See EPAPS supplementary material at http://dx.doi.org/10.1063/1.3264073 for mechanical drawings of custom-fabricated parts and annotated technical photographs.

4 i.e., place both phases in the sample tube before actuating the vortexer and initiating vaporization.

5 This was in order to minimize the possibility of forming artifactual lipid structures in the presence of high local concentrations of solvent, among other reasons. See Ref. 1.


11 As well as that the fast-phase solvent fraction represents less than 2% of $A_0$ [i.e., $(l_1-k_1)/(l_2-k_1) \approx (l_2-l_1)/(l_2-l_1)]$.

12 The original device yielded $\lambda_1 \approx 0.2$ s$^{-1}$ for chloroform at room temperature. See Fig. 6, Ref. 1.