Cytomimetic Modeling in Which One Phospholipid Liposome Chemically Attacks Another

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Cytomimetic Modeling in Which One Phospholipid Liposome Chemically Attacks Another

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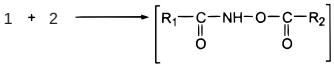
Widespread membrane/membrane reactivity in biology (e.g., in viral attack upon cells and in sperm penetration of ova)[1] would lead one to expect considerable attention directed toward simple "cytomimetic"[2] models of such processes. This has not, however, yet occurred. For example, the kinetics of bond-forming reactions between two populations of liposomes (hollow spheres comprised of bilayer membrane shells) are seemingly unknown.[3,4] Studies have been mainly confined to the bimolecular kinetics of species in solution reacting with a species adsorbed onto a liposome.[5−8] Thus, dithionite ($S_2O_4^{2-}$) dissolved in water reduces a liposome-bound fluorescent dye; the ensuing loss of fluorescence provided rates of the solution/liposome reaction.[9] On a more biological level, it has been shown that water-soluble enzymes (chymotrypsin and acetylcholineesterase) catalyze the hydrolyses of liposomal substrates at rates that depend on the ability of the substrates to project themselves beyond the membrane surface.[10] It is the purpose of the present work to examine liposome/liposome chemistry and to compare it with the corresponding solution/solution, solution/liposome, and intra-liposome reactions. The variations are all depicted in Figure 1.

Liposomal reactions were made possible by endowing one set of liposomes with nucleophilicity (via a hydroxamate) and another set of liposomes with electrophilicity (via a *p*-nitrophenyl ester) using the compounds in Figure 2.[11]



(1)

Under the basic conditions of our experiments (pH 9.0), the acylhydroxamate intermediate rapidly hydrolyzes to regenerate hydroxamate, thereby allowing for turnover and no net consumption of nucleophile. The byproduct, *p*-nitrophenolate, has a strong absorbance at 400 nm from which the rate of reaction can be determined.

Nucleophilic and electrophilic entities were incorporated into liposomal membranes via a cholesterol unit derivatized at its 3-position as shown in compounds **1** and **2a**, **2b**, **2c**, respectively (Figure 2). Electrophiles **2a**, **2b**, and **2c** differ only in the length of their hydrophilic spacers separating the ester functionality from the cholesterol moiety. Cholesterol was selected as the liposomal "anchor" owing to its known affinity for bilayer membranes.[12]

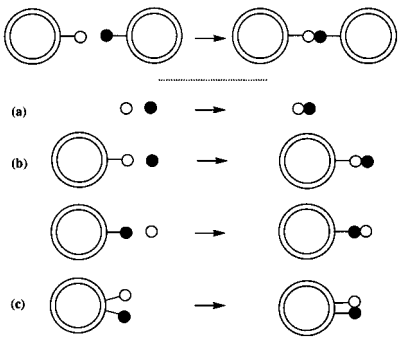


Figure 1. Schematic representation of an inter-liposomal reaction compared to (a) solution/solution, (b) solution/liposome, and (c) intra-liposomal reactions.

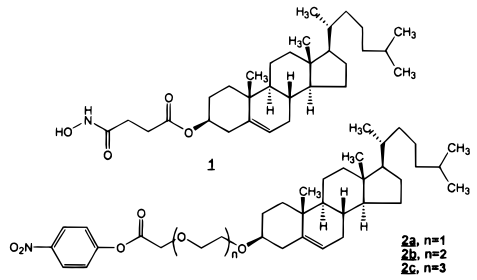


Figure 2. Structures of nucleophile **1** and electrophiles **2a**, **2b**, and **2c** that were used for surface modification of the liposomes.

Liposomes were prepared by hydrating for about 5 min at room temperature a cast film composed of 2 mg of phospholipid (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) plus 10 mol % of **1** or **2** (0.15−0.20 mg) in 1 mL of gently stirred buffer. The resulting suspension was passed back and forth 19 times through a polycarbonate membrane (100 nm pore diameter) with the aid of a LiposoFast vesicle extruder.[13] The liposomes had mean hydrodynamic diameters of 120 ± 15 nm (measured routinely with a Coulter N4 particle sizer before and after the kinetic runs). A liposome preparation was diluted with buffer, mixed with a water-soluble reagent or second liposome preparation, and assayed at 400 nm and 25.0 °C for ester hydrolysis.

Note that addition of **1** or **2** (as an acetonitrile solution) to preformed liposomes composed of pure phospholipid is an unsatisfactory means of fabricating chemically reactive liposomes because the additives precipitate in water before they have a chance to enter the membrane. Owing to this water insolubility of **1** and **2**, no chemical reaction can occur between them in aqueous buffer. This fact, plus the normal translucency of the liposome preparations obtained from **1** or **2** and phospholipid, establish that the reactants are membrane-bound.

Before describing liposome/liposome reactivity, it will be helpful to discuss, by way of calibration, the rates of the simpler systems in Figure 1. *p*-Nitrophenyl acetate (pNPA), at pH 9.0 and 25.0 °C in the absence of any nucleophile, hydrolyzes "spontaneously" with an observed rate constant of only 2.0×10^{-2} min^{-1} (half-life $t_{1/2} = 35$ min). A mixture of pNPA (1.0×10^{-4} M) and acetohydroxamate AH (1.0×10^{-3} M) under the same conditions gave an observed rate constant $k_{obs} = 2.0$ min^{-1} ($t_{1/2} = 0.35$ min). These values agree with literature data.[14]

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Table 1. Summary of Rates for Reactions in Figure 1[a]

	10^3[hydroxamate], mol/L[b]	10^4[ester], mol/L[c,f]	pH	k_{obs}, min^{-1} [d]	$t_{1/2}$, min
pNPA, buffer		1.0	9.0	2.0×10^{-2}	35
AH + pNPA	1.0	1.0	9.0	2.0	0.35
$\mathbf{2a}_{ves}$, buffer		1.0	9.0	3.8×10^{-3}	180
AH + $\mathbf{2a}_{ves}$	1.0	1.0	9.0	9.0×10^{-2}	7.7
AH + $\mathbf{2a}_{ves}$	0.10	1.0	9.0	1.8×10^{-2}	38
$\mathbf{1}_{ves}$ + pNPA	0.10	0.50	9.0	9.8×10^{-2}	7
$\mathbf{1}_{ves}$ + $\mathbf{2a}_{ves}$	0.10	0.50	9.0	5.8×10^{-3}	120
$(\mathbf{1} + \mathbf{2a})_{ves}$	0.10	0.50	8.0[e]	2.1 (stage 1)	0.3
				0.12 (stage 2)	6

[a] All measurements were performed at 25 ± 0.3 °C in 0.1 M carbonate or 0.1 M phosphate buffer. [b] Concentration of AH or liposome-bound **1**. [c] Concentration of pNPA or liposome-bound ester **2a**. [d] Rate constants are reproducible to ±10%. [e] Reactions were too fast to follow at pH 9.0. [f] **2a**, **2b**, and **2c** all react at similar rates.

When ester **2a** is bound to the liposomes, it hydrolyzes more slowly under the standard conditions than does "free" pNPA. Thus, "spontaneous" hydrolysis of liposomal **2a** in the absence of nucleophile has a $t_{1/2}$ of 180 min instead of the 35 min for aqueous pNPA. The rate decrease also manifests itself in the bimolecular reaction between liposomal ester (1.0×10^{-4} M) and solution-phase AH (1.0×10^{-3} M) where $t_{1/2}$ increases from 0.35 min for the solution/solution reaction to 7.7 min for the corresponding solution/liposome reaction. Clearly, accessibility factors at the membrane surface help protect the ester from nucleophilic attack by external AH. This could likely occur by partial burying of the ester group via looping of the spacer separating it from the embedded cholesterol moiety.[15,16]

It was also of interest to interchange reactants in the solution/liposome reaction by placing hydroxamate **1** in the liposome and the ester (pNPA) in the external water. We needed here to reduce the hydroxamate concentration 10-fold to 1.0×10^{-4} M in order not to overload the liposome with more than 10 mol % of guest nucleophile. In any event, these liposomes reacted with 0.5×10^{-4} M pNPA (a cleanly first-order process characteristic of turnover) at a rate of 9.8×10^{-2} min^{-1} ($t_{1/2} = 7$ min). If this rate is elevated 10-fold to adjust for the lower hydroxamate concentration relative to that of the solution/solution reaction, then one sees that the liposomal rate is only a factor of 2 less than that of the nonvesicular AH + pNPA reaction. In other words, liposomal nucleophile and "free" nucleophile have similar reactivities (in contrast to the situation with liposomal and "free" ester). The difference between hydroxamate and ester must be that the former is anionic and, therefore, the hydroxamate of liposomal **1** tends to remain fully exposed to the bulk water.

We are now in position to discuss the liposome/liposome reaction carried out with 1.0×10^{-4} M **1** in one set of liposomes and 0.5×10^{-4} M **2a** in another. The observed rate constant of 5.8×10^{-3} min^{-1} ($t_{1/2}$=120 min) is similar to, but definitely faster than, "spontaneous" buffer hydrolysis of liposomal **2a** (which, as mentioned, has a $t_{1/2}$=180 min). Multiple repeat runs ensure that the liposome/liposome chemical reaction is real and, to our knowledge, the first instance of its kind. At this point it is not possible to specify the number of hydrolytic events per liposome/liposome collision.

Dynamic light scattering experiments upon completion of the liposome/liposome reactions showed no significant particles larger than the original 120 nm liposomes. This is consistent with turnover, *i.e.* two liposomes will chemically couple to each other after which the acyl intermediate hydrolyzes, the liposomes become quickly independent once again, and the hydroxamate concentration remains constant.

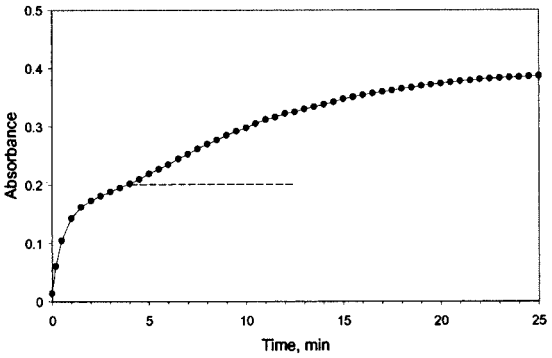

Figure 3. Absorbance at 400 nm for the biphasic **1** + **2a** intra-liposomal reaction (pH 8.0, 25.0 °C). The horizontal dashed line represents an approximate "infinity" for the first stage involving reaction within the outer leaflet of the membrane bilayer.

By far the fastest membrane reaction occurred when the nucleophile and electrophile (at comparable concentrations) were contained within one and the same bilayer. In fact, the intra-liposomal reaction was so fast that we had to make two adjustments to our procedure: (a) Since hydration and extrusion of a phospholipid film containing **1** and **2** gave immediate hydrolysis under basic conditions, the film hydration was carried out at pH ~3. Extrusion of the acidic suspension produced the liposomes. (b) Reactions were then initiated by rapidly bringing the pH up to 8.0 (not the usual 9.0). A one-unit lowering of the pH slowed the reaction by reducing roughly 8-fold the percent of hydroxamate in the hydroxamic acid/hydroxamate mixture ($pK_a = 9.4$).[17]

A plot of *p*-nitrophenolate absorbance at 400 nm vs time shows a decidedly biphasic behavior (Figure 3) when **1** (1.0×10^{-4} M) and **2a** (0.5×10^{-4} M) share a common membrane. This is most simply interpreted as an initial fast intra-liposomal reaction ($t_{1/2}$ = 0.3 min) between **1** and **2a** within the outer leaflet of the phospholipid bilayer. As the conjugate acid form of **1** in the inner leaflet is converted into hydroxamate via inward diffusion of hydroxide through the bilayer, or as ester **2a** flip-flops from the inner to outer leaflets, the remaining half of the reaction can proceed. This second and slower component of the biphasic process has a half-life of about 6 min. Note that the chemistry is proceeding here at a pH of only 8.0 and 0.1 mM hydroxamate. Adjusting the rate constants upward (to correct for the lower pH and for the lower hydroxamate concentration relative to that of the AH/pNPA reaction) leads to the conclusion that the intra-liposomal reaction is roughly 1−2 orders of magnitude faster than even the solution/solution reaction. Confinement of the bimolecular reaction to the membrane obviously has a highly positive effect upon the kinetics. Indeed, among the membrane reactions only the intra-liposomal mode is sufficiently fast to allow detection of accompanying diffusion-controlled processes.

In summary, we have determined that several modes of liposomal reactivity under comparable conditions have rates of decreasing magnitude according to the following: intra-liposome > solution/solution > solution/liposome > inter-liposome. It appears as if reactivity of a membrane surface depends markedly upon the affinity of the relevant functionalities for the aqueous medium. In our case, one functionality (the hydroxamate) is fully exposed, whereas the other (the ester) is not, a situation that leads to a slow but observable liposome/liposome reaction.

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Supporting Information Available: Experimental details, characterization of compounds, and additional kinetic data for **2b** and **2c** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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