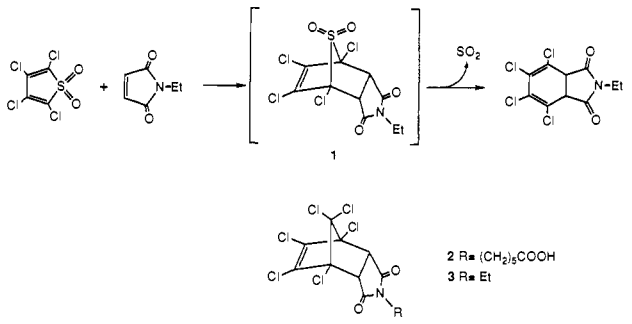

Figure 1.

However, the reaction product is not an appropriate hapten for generating catalytic antibodies, since severe product inhibition would be expected to prevent efficient turnover of the catalyst. An alternate strategy is shown in Figure 1. Tetrachlorothiophene dioxide (TCTD) reacts with *N*-ethylmaleimide (NEM) to give an unstable, bicyclic intermediate **1** that subsequently extrudes SO_2, to give a dihydrophthalimide as product.[9] We reasoned that a stable analogue of the bicyclic adduct could elicit an antibody combining site with the proper shape for promoting the target reaction. As the final product does not closely resemble the transition state of the reaction, product inhibition would be minimized, allowing multiple turnovers of the catalyst.

To test this notion, we prepared five high-affinity monoclonal antibodies against hapten **2**,[10,11] a stable analogue of bicyclic adduct **1**. Because TCTD reacts with lysine residues on the surface of immunoglobulins, it was necessary to reduce the nucleophilicity of the amino groups by exhaustive reductive methylation with formaldehyde and sodium cyanoborohydride.[12] TCTD was shown to be stable in the presence of the methylated antibodies which also retained high affinity for the hapten as judged by ELISA.[13] Chemical modification of immunoglobulins in this way is likely to be of general value, as it will allow reactive molecules like epoxides, Michael acceptors, or other alkylating agents to be employed as substrates for catalytic antibodies.

The methylated antibodies were assayed at 25 °C for their ability to promote the Diels-Alder reaction between TCTD and NEM in aqueous buffer (20 mM MES, 100 mM NaCl, pH 6.0) containing 10% acetonitrile. Cycloadditions were followed by monitoring the disappearance of TCTD at 330 nm and by analytical reverse-phase HPLC.[14] The products of the reaction, dihydro-*N*-ethyltetrachlorophthalimide and the fully oxidized *N*-ethyltetrachlorophthalimide, were isolated and characterized. Sulfur dioxide was detected independently by bleaching of malachite green at 617 nm. In the preliminary assays, one antibody (secreted by hybridoma 1E9) significantly accelerated the rate of reaction over the uncatalyzed process and was characterized further.

Antibody 1E9 promotes the target reaction with multiple (>50) turnovers, and several lines of evidence demonstrate that the observed catalysis is not artifactual. The process is first order with respect to immunoglobulin concentration, and its substrate specificity matches expectations based on hapten structure. Thus, *N*-ethylmaleimide is a good substrate for the catalyst, but maleimide is not. Also, the catalyzed reaction is strongly inhibited by compound **3**,[10] a close analogue of the hapten used for immunization. Preincubation of the antibody (5 μM) with an equimolar amount of **3** stops the catalyzed reaction completely, even at concentrations of maleimide 10^3-fold greater than the concentration of inhibitor. Finally, methylated antibodies other than 1E9 fail to catalyze the cycloaddition.

When initial rates with the antibody were measured as a function of NEM concentration, the concentration of TCTD being held constant, saturation kinetics were observed. At 0.61 mM TCTD, for example, the apparent values of k_{cat} and $(K_m)_{NEM}$ were 4.3 ± 0.3 min^{-1} and 21 ± 4 mM, respectively. Low solubility of TCTD prevented determination of its K_m value and, hence, the true k_{cat} for the reaction. Nevertheless, comparison of $(k_{cat})_{app}$ obtained at 0.61 mM TCTD with the second-order rate constant for the uncatalyzed cycloaddition (0.040 ± 0.007 min^{-1}) yields an apparent effective molarity of at least 110 M/binding site. Because $(k_{cat})_{app}$ is linearly dependent on TCTD concentration in this range, the true effective molarity must be substantially higher than this value.

These experiments provide the first example of an antibody-catalyzed Diels-Alder cycloaddition and demonstrate the feasibility of using antibody technology to promote important nonphysiological reactions. Particularly noteworthy aspects of this study include the following: (1) the design of the hapten so as to minimize product inhibition, and (2) the use of chemically modified antibodies to permit study of reactive substrate molecules. We are currently extending these concepts to other [2 + 4] cycloadditions. Tailored "Diels-Alderase" antibodies will be particularly valuable as tools for studying proximity effects in catalysis and for effecting regio- and stereoselective transformations in organic synthesis.

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(11) The *N*-hydroxysuccinimide ester of hapten **2** was coupled to keyhole limpet hemocyanin, and the resulting hapten-protein conjugates were used to generate an immune response in 129 GIX$^+$ mice. Standard protocols were used to fuse mouse spleen cells with SP2/0$^+$ myeloma cells.[4a] IgG antibodies specific for the hapten were isolated and purified as previously described.[4a] Proteins were judged >95% pure by sodium dodecylsulfate polyacrylamide gel electrophoresis with Coomasie blue staining. Antibody concentration was determined from A_{280} ($\epsilon^{0.1\%} = 1.40$ mL mg^{-1} cm^{-1}) and a molecular weight of 160 000.
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Photoinduced Destabilization of Bilayer Vesicles

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The polymerization of lipid bilayer vesicles (liposomes) is an effective method to enhance the long-term colloidal and chemical stability of the aqueous suspensions.[1] A host of methods to polymerize vesicles have been described since the first reports in the early 1980s.[2] It was soon recognized that the polymerization of two-component vesicles, where only one component was polymerizable, resulted in phase separation of the lipids into polymeric and monomeric domains.[3-5] The polymerizable lipids form

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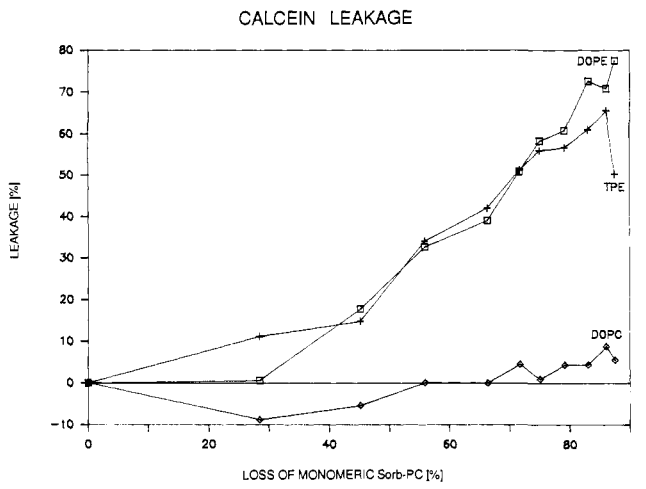
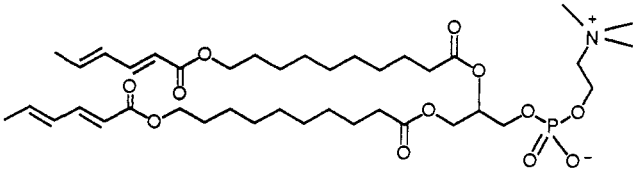


Figure 1. Leakage of calcein at 24 °C from vesicles containing 75 mM calcein vs the loss of monomeric SorbPC during photopolymerization. The percent leakage was calculated from the fluorescence intensity at 550 nm (footnote 16). The vesicles were prepared in a 2/1 molar ratio of DOPE/SorbPC; TPE/SorbPC; and DOPC/SorbPC and desalted by SEC with 150 mM NaCl, 2 mM imidazole, 2 mM 2-{[tris-(hydroxymethyl)methyl]amino}ethanesulfonic acid at pH 7.4.

covalently linked domains as the reaction proceeds, which in turn produces domains of the nonpolymerizable lipid. If both components are lipids, e.g., phosphatidylcholines (PC), which prefer lamellar structures, the polymerization proceeds with retention of vesicle integrity without loss of encapsulated aqueous contents. However, if the nonpolymerizable lipid can assume a nonlamellar phase, the polymerization-induced phase separation leads to vesicle destabilization with the concomitant release of the aqueous contents. Here we describe the efficient photoinduced destabilization of bilayer vesicles, which offers a spatially and temporally selective method of release of chemical or biological reagents.

In order to illustrate this phenomenon, consider the properties of the following two-component vesicle bilayer preparations:[6] (a) dioleoylphosphatidylethanolamine (DOPE)/SorbPC (2/1 molar ratio), (b) TPE/SorbPC (2/1 molar ratio) (TPE is a PE prepared by transphosphatidylation of egg PC), and (c) dioleoylPC (DOPC)/SorbPC (2/1 molar ratio). Bilayer membranes of the polymerizable SorbPC in water absorb at 257 nm[7,8] and are readily



SorbPC

polymerized by irradiation with 254-nm light (low-pressure mercury lamp). Phosphatidylethanolamines, e.g., DOPE and TPE, can form nonlamellar structures under physiological conditions,[9] and therefore PEs are likely to be important in the membrane processes of fusion, endo- and exocytosis, and the transmembrane movement of large molecules. Vesicles from PEs are not stable at physiological pH,[10] unless they are combined with other lipids such as PC. Processes that lead to phase separation of PE and other lipids, such as PC, can trigger the PE lamellar to nonlamellar phase transition.[11-14]

The membrane permeability of these vesicles was determined as a function of the extent of monomer (SorbPC) polymerization via the self-quenched calcein assay[15] (Figure 1). The vesicles were formed by hydration and sonication in a buffer containing the aqueous marker and then desalted by size exclusion chromatography (SEC). An increase in membrane permeability enhances the sample fluorescence. The measured sample fluorescence was compared to the fluorescence of 100% dye release (achieved by Triton X-100 lysis) to yield the percent leakage.[16] The data shown in Figure 1 indicates that the leakage was insignificant for the photolyzed DOPC/SorbPC vesicles, whereas the DOPE/SorbPC and the TPE/SorbPC vesicles become permeable after 30–50% loss of monomer. This corresponds to 1–2-min continuous exposure to the UV light at an absorbed flux of about 5×10^{14} photons/s.

The photoinduced destabilization of the PE/SorbPC vesicles was verified with a second permeability assay based on the coencapsulation of a fluorescent dye and its quencher.[11] Photopolymerization of the PE/SorbPC (but not DOPC/SorbPC) vesicles lead to leakage, dye-quencher separation by dilution, and fluorescence enhancement.

The photoinduced destabilization of PE/SorbPC vesicles and the release of aqueous markers appear to require two processes: (a) lateral phase separation of the two membrane components driven by the photopolymerization reaction and (b) a propensity of the nonpolymerizable lipid to assume a nonlamellar phase as enriched domains of this lipid are formed. Formation of a nonlamellar phase is suggested by the increase in fluorescence of the membrane probe, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE,[17] upon photopolymerization of PE/SorbPC (but not DOPC/SorbPC) vesicles.[6] Preliminary ^{31}P NMR data indicate that this phase is isotropic rather than hexagonal.[18] Some PE membranes display an isotropic phase in the temperature range immediately below the onset of the hexagonal phase transition, T_H.[19] The values of T_H for model membranes of DOPE/DOPC decrease with decreasing DOPC content.[20] Thus the photopolymerization reaction of PE/SorbPC initiates a change in the monomer composition of the membrane, e.g., 50% polymerization leaves a 4/1 ratio of DOPE to monomer PC. This change in composition lowers the temperature for the transition(s) from lamellar to nonlamellar phases to near that of the ambient conditions and permits the formation of nonlamellar phases. The cooperative effect of the lipids at the phase transition amplifies the perturbation initiated by the absorbed light and leads to an efficient lysis of the bilayer vesicles.

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