BULLETIN
OF THE
KOREAN CHEMICAL SOCIETY

VOLUME 17, NUMBER 10
OCTOBER 20, 1996

BKCS 17(10) 875-974
ISSN 0253-2964

Communications

pH-Sensitive Liposomes Containing a Bipolar Amphiphile as a Protonatable Component

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Received May 27, 1996

Immunoliposomes were developed to facilitate association of liposomes with target cells by using cell-specific ligands attached to the liposome surface.[1] The conventional immunoliposomes uptake following target cell binding *via* receptor-mediated endocytosis is delivered to lysosomes where phospholipases disrupt liposomal bilayers and entrapped drugs are released.[2] Many drugs, however, are susceptible to the lysosomal degradation. pH-Sensitive liposomes were designed to circumvent the lysosomal catabolic degradation by delivering encapsulated drugs at a pre-lysosomal site. This class of liposomes are generally composed of unsaturated phosphatidylethanolamine and a weakly acidic amphiphile. Examples of the acidic amphiphiles are fatty acids,[3,4] fatty acyl amino acid,[5] cholesterol hemisuccinate[6] and diacylsuccinylglycerol (DASG).[7,8] Interestingly, liposomes composed of diacetylenic phosphatidylethanolamine, fatty acid, and nonpolymerizable phosphatidylethanolamine after polymerization with UV light at 0 ℃ retained the initial pH-sensitivity and became more stable than monomeric liposomes.[9] When pH-sensitive liposomes are exposed to endosomes at pH 5.0-6.5, a liposome-endosome fusion occurs with the release of drugs into the cytoplasm. The pH-sensitivity of these liposomes is primarily due to a phase transition to the nonbilayer phases.[10,11]

We report that liposomes containing a membrane-spanning bipolar amphiphile, di(6-hemisuccinyloxyhexyl) muconate (DHM),[12] are pH-sensitive. In these liposomes DHM acts as a protonatable component. DHM does not form monolayer vesicles by itself as expected because of the difference in the outer and inner radius curvature of the vesicles.[13,14] Hydration of a mixture of DHM and dioleoylphosphatidylethanolamine (DOPE) or cholesterol did not form vesicles.

Lipid films composed of DOPE, dioleoylphosphatidylcholine (DOPC) and DHM in a 3 : 3 : 1 molar ratio were hydrated by repeated freeze-thawing[15] in a 50 mM calcein phosphate-buffered saline (PBS, pH 8.0) solution, and then sonicated with a Cole-Parmer tip type sonicator. The suspension was chromatographed on Bio-Gel A 0.5 m column equilibrated with PBS at pH 7.5 to remove unentrapped calcein. The calcein solution was made isotonic to PBS of 300 mosm/kg by adding NaCl. After dissolving lipid in methanol, the concentration after chromatography was measured by the UV absorbance of DHM at 264 nm (ε 31100).

Liposomes were incubated in PBS solutions of various pH at 37 ℃ for 1 h, and then adjusted to pH 7.5 by adding an appropriate amount of a dilute HCl solution. The calcein fluorescence intensity was measured with a FD-110 JASCO spectrofluorometer at excitation and emission wavelengths of 490 and 518 nm, respectively. Percent release of liposomal calcein was calculated from the following formula:

$$\% \text{ Release} = \{(F - F_o)/(F_i - F_o)\} \times 100$$

where F_o = fluorescence intensity of liposomes in PBS at pH 7.5, F = fluorescence intensity after incubation and F_i = fluorescence intensity after the addition of Triton X-100.

The liposomes entrapped calcein with a fluorescence quenching of 80%, indicating that the calcein concentration in the liposomes was approximately 50 mM.[8] The liposome mean diameter was estimated to be 100± 15 nm by quasi-elastic laser light scattering (BI-8000AT Brookhaven).[16] Transmission electron microscopy revealed that the liposomes were unilamellar. There was no appreciable release of

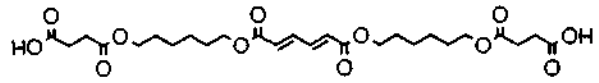

Figure 1. Chemical structure of di(6-hemisuccinyloxyhexyl) muconate (DHM).

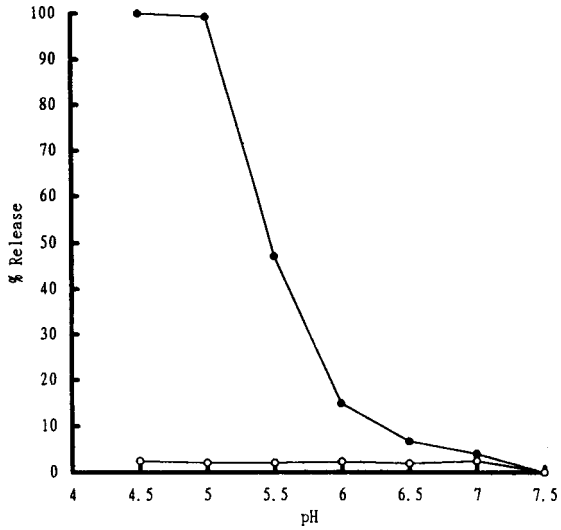

Figure 2. Percent calcein release from liposomes composed of DOPE/DOPC/DHM (3/3/1) (●) and DOPE/DOPC (1/1) (○) at various pH.

calcein from liposomes during their storage at −4 ℃ for several days. While these liposomes are stable at neutral pH, they undergo destabilization in acidic conditions. The liposomes incubated at 37 ℃ in PBS of pH 4.8 and 5.8 had increased fluorescence intensity that reached a plateau within 30 and 50 min, respectively. Half of the entrapped calcein was released from the liposomes near pH 5.5 as shown in Figure 2. On the other hand, liposomes composed of only DOPE and DOPC in a 1 : 1 molar ratio were not destabilized at the weakly acidic pH. This result indicates that DHM in the liposomes acts as an acidic component.

The liposomes must be stable in the blood for them to be used as *in vivo* drug carriers. Although liposomes combined with DOPE and a single-chain amiphiphile such as oleic acid are pH-sensitive, they are not stable enough to use *in vivo* because the acidic components are rapidly extracted from the liposome membrane by serum proteins.[17,18] However, small pH-sensitive liposomes composed of DOPE and a double-chain amphiphile such as DASG are less permeable in plasma.[7]

The stability of the pH-sensitive liposomes containing DHM was examined in 90% rabbit plasma at 37 ℃ by measuring the release of entrapped calcein as a function of time. Less than 10% entrapped calcein was released at the end of a 2 h incubation. This result indicates that these liposomes are quite stable in plasma. Small liposomes composed of DOPE and DASG release entrapped calcein 5-20% after a 2 h incubation in 90% human plasma.[7,8,17] Thus, the plasma stability of this new pH-sensitive liposomes prepared from the membrane-spanning acidic DHM may be comparable to that of the conventional pH-sensitive liposomes containing the double-chain acidic components.

To remove a membrane-spanning bipolar amphiphile embedded in liposome membranes, one must remove water molecules associated with the inner hydrophilic headgroup and push it through the hydrophobic membrane core.[19] This process is energetically unfavorable. A single-chain bipolar amphiphile may assume the extended transmembrane arrangement or U-shaped conformation as represented in Figure 3.[20] Any bipolar amphiphile with the bent conformation located in the outer leafleat of liposome membranes will be readily extracted by serum proteins. However, the muconyl group in DHM, the rigid segment, is expected to prevent the amphiphile from being bent (U-shaped) in the liposome membrane. This further protects the acidic component from extraction in plasma.

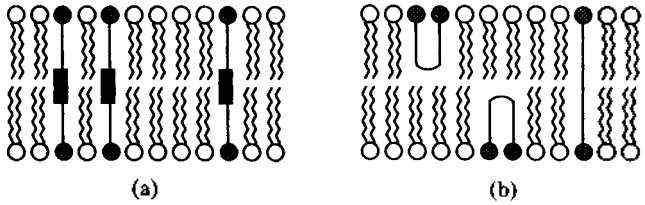

Figure 3. Schematic representation of lipid bilayers containing bipolar amphiphiles (a) with or (b) without a rigid segment.

This initial study opens the possibility for the use of weakly acidic bipolar amphiphiles in preparing pH-sensitive liposomes. The mechanism of the acid-induced destabilization of these liposomes are under investigation.

Acknowledgment. This work was supported by a grant from KOSEF (951-0305-1). We thank Professor Sung-Sik Kim (Chonbuk National University) for the usage of the FD-110 JASCO spectrofluorometer and Professor Jong-Duk Kim and Mr. Jin-Chul Kim (Korea Advanced Institute of Science Technolgy) for the light scattering experiment.

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12. Muconic acid dichloride prepared by refluxing muconic acid in thionyl chloride underwent esterification with 1,6-hexanediol in the presence of pyridine in tetrahydrofuran to yield di(6-hydroxyhexyl) muconate which was then coupled with succinic acid anhydride in boiling toluene to produce DHM as a white solid. mp 99-100 ℃ (uncorrected). Analysis. calcd. $C_{26}H_{38}O_{12}$: C, 57.5; H, 7.06%. Found: C, 56.7; H, 7.02%. ^{1}H NMR ($CDCl_3$): δ 1.6 (m, 16H, CH_2), 3.6 (t, 4H, CH_2-O), 4.2 (t, 4H, CH_2-O-C=O),

6.2-6.3 (m, 2H, CH=CH-CH=CH), 7.2-7.3 (m, 2H, CH =CH-CH=CH). UV: λ_{max}=264 nm, methanol, ε=31100. Differential Scanning Calorimetry (DSC): main phase transition temperature=79 ℃ at the maximum excess heat capacity in PBS.

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The Relative Reactivity of Palladium towards Migration and Ring-Opening of Three-and Four-Membered Ring Alkanes and Ethers

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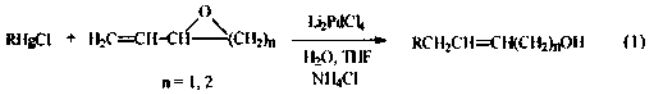
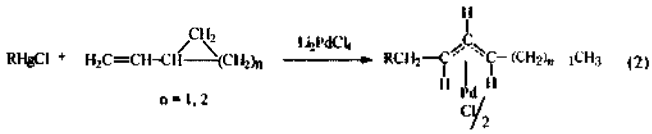
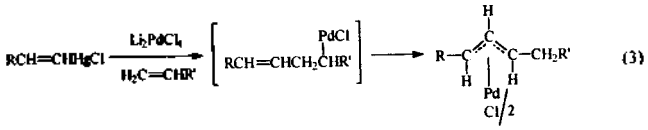
Received June 15, 1996

Vinylcyclopropanes[1] and methylene cycloparopanes[2] have been ring-opened by palladium(0) reagents and palladium dichloride. Vinylic epoxides[3] and oxetanes[4] also undergo palladium(0) catalyzed ring-opening reactions useful in organic synthesis. We and others have recently observed that the addition of organopalladium compounds to unsaturated epoxides[5] and oxetanes,[6] as well as unsaturated cyclopropanes and cyclobutanes,[7] leads via facile ring-opening to high yields of unsaturated alcohols and π-allylpalladium compounds respectively (eqs. 1, 2). Since the mechanism we proposed for

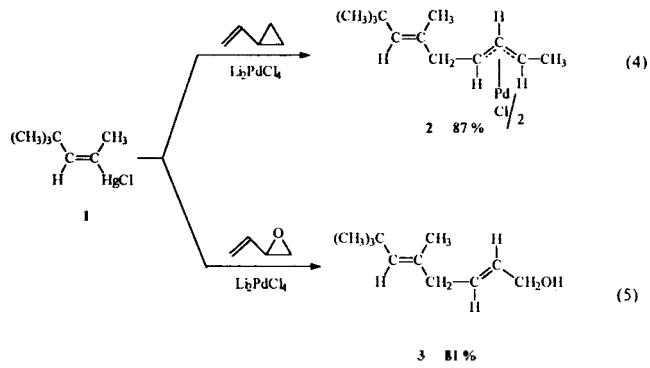


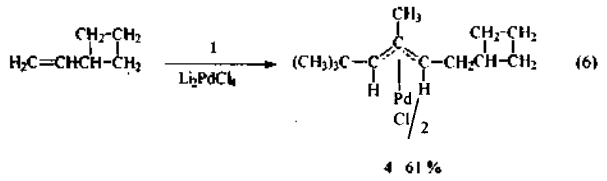


these processes had little literature precedent,[2h-j,7c,8] we sought further information on these reactions. In conjunction with our earlier reported method for the preparation of π-allylpalladium compounds from Li_2PdCl_4, vinylmercurials and alkenes (eq. 3),[9] we have now studied the relative reactivity of palladium towards migration (eq. 3) and the ring-opening of three-and four-membered ring alkanes and ethers (eqs. 1, 2).



Vinylmercurial **1** reacts with Li_2PdCl_4 and vinylcyclopropane or 3,4-epoxy-1-butene to give predominantly the corresponding ring-opened π-allylpalladium compound **2**[7] and dienol **3**[5] respectively (eqs. 4, 5), while vinylcyclobutane affords only the migration product **4** (eq. 6). X-ray crystallographic analysis[10] of dimer **4** (Figure 1) indicates a crystallographic 2-fold axis passing through the chlorine atoms. This is the first example, to our knowledge, of such symmetry in a π-allylpalladium dimer. All bond angles and distances are within the range expected. Compound **4** adopts a "transplanar" arrangement in which the cyclobutyl rings appear to be only slightly puckered (± 0.06 Å)





Vinyl oxetane **5** has been observed to afford comparable amounts of ring-opened dienol **6** and π-allylpalladium migration product **7** (eq. 7)[6]

Consistent with the higher reactivity of the cyclopropane versus the cyclobutane in vinylcyclopropane and vinylcyclobutane, the reaction of phenylmercuric chloride, Li_2PdCl_4 and 1-cyclobutyl-1-cyclopropylethene afforded only a syn-anti mixture of the cyclopropane-opened products **8** (eq. 8).

We have established the ability of palladium to migrate prior to ring-opening by obtaining π-allylpalladium compound **9** from the reaction of phenylmercuric chloride, Li_2PdCl_4 and allylcyclopropane (eq. 9). On the other hand, the reaction of vinylmercurial **1**, Li_2PdCl_4 and allylcyclopropane gives two