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Non-peptidic liposome-fusion compounds at acidic pH

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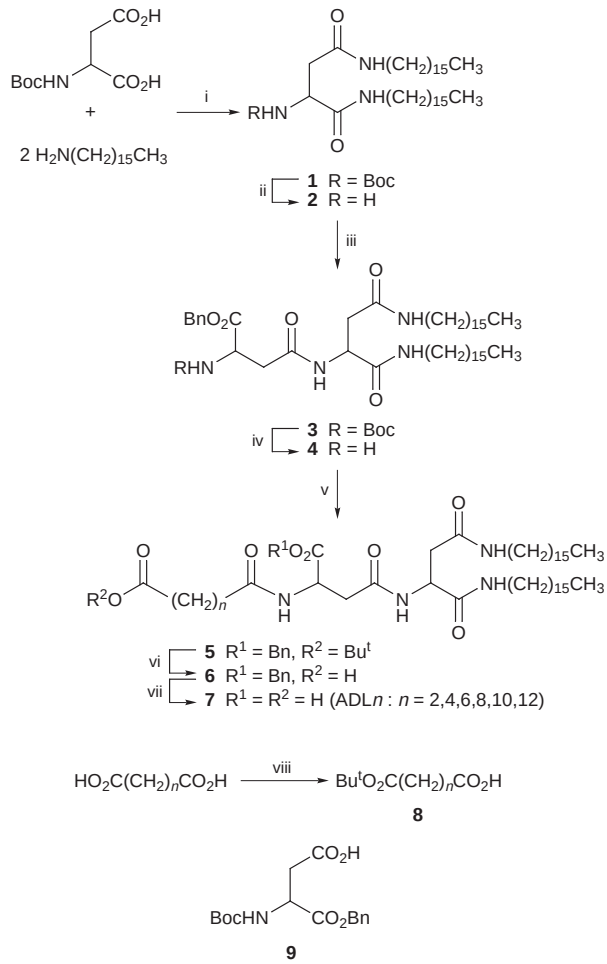
Liposomes including aspartic acid-derived artificial lipids (ADL) with various carboxy alkyl chains as head groups (ADL*n*; *n* indicates the number of the methylene groups, *n* = 2,4,6,8,10,12) are prepared, in which ADL6 and ADL8 liposomes induce remarkably high lipid-mixing in the acidic region.

For the last decade, liposomes have been exploited predominantly as potent carriers of various polar materials into cells.[1–4] We show here that artificial lipids with simple structures can induce pH-dependent membrane fusion in a manner similar to authentic fusion inducers such as the Spike protein of the influenza virus.[5–7] Homologous artificial lipids (ADL*n*; *n* = 2, 4, 6, 8, 10, 12) were designed and synthesized according to Scheme 1.† These lipids are composed of three fundamental parts: (A) two long alkyl chains in the tail part, which can be anchored to the hydrophobic region of lipid bilayer, (B) a polar moiety composed of two aspartic acid residues in the central part, (C) a terminal carboxylic acid group as a pH-dependent trigger for liposome fusion. Part (C) composed of the carboxy group and the alkyl chain was designed so that its polarity could be controlled by pH, depending upon whether the carboxy group is protonated or deprotonated. Since the pK_a was expected to be 4.6–4.8, in view of the pK_a of an ordinary carboxy group, part (C) was expected to behave as a hydrophobic protrusion on the lipid membrane like fusion peptides[8] at acidic pH. The time course of fusion % of liposomes composed of ADL*n* (5–20 mol%) and egg phosphatidylcholine (Egg PC, Avanti Polar Lipids, Inc., special grade) was measured from the efficiency of the fluorescence resonance energy transfer (FRET) between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and lissamine rhodamine B sulfonyl phosphatidylethanolamine (Rh-PE).[9,10]‡ The fusion % was calculated from eqn. (1),

$$\text{Fusion \%} = 100[F(t)-F(0)]/[F(\text{Triton})-F(0)] \qquad (1)$$

where $F(0)$, $F(t)$ and F(Triton) signify the fluorescence intensity at 530 nm (λ_{ex} = 480 nm) before adding citric acid, at time t, and after the collapsing of liposomes by Triton X-100, respectively. Fusion % after 5 min reaction at pH 4 is depicted in Fig. 1 ([ADL*n*] = 5, 10, 15, 20 mol%). As expected, very high liposome fusion was observed in some liposomes, which suggests that the carboxy group in the head part can act as a trigger in the fusion. It should be noted here that there are optimal lengths of the methylene group in the head group between 6 and 10, with ADL6 (20 mol%) showing especially high fusion % (*ca.* 40%). It was also confirmed that at neutral pH the liposome fusion was scarcely observed for any of the lipids, at least within 1 h. The pH dependence of the fusion % for ADL6 and ADL8 is shown in Fig. 2 together with the control experiment where liposomes are composed of only Egg PC without ADL*n*. The fusion in the presence of ADL6 or ADL8 was significantly accelerated below *ca.* pH 5 due to the protonation of the carboxy group, while that of the control liposome gave low fusion % between pH 3.6 and 7.2. In contrast to ADL6, ADL8 and ADL10, the lipids with shorter or longer chains, ADL2, ADL4 and ADL12, did not induce remarkable fusion even at acidic pH.

In most fusion inducers such as the Spike protein of the influenza virus, the hydrophobic fusion peptide is hidden at neutral pH. At acidic pH, however, it protrudes from the membrane surface and is incorporated into another membrane. Consequently these membranes are close together, resulting in membrane fusion. The carboxylic acid with a long alkyl chain, which is protonated at low pH, seems an appropriate hydrophobic group to mimic fusion peptides as a simple fusion inducer. Fig. 2 clearly suggests that the protonation of the terminal carboxylate of ADL*n* is the trigger of liposome fusion and Fig. 1 suggests that there is an optimal hydrophobicity



Scheme 1 Synthetic route to ADL*n*: i, HOBt, TBTU, DMAP; ii, TFA; iii, **9**, HOBt, EDC, DMAP; iv, TFA; v, **8**, HOBt, TBTU, DMAP; vi, TFA; vii, H_2/Pd-C; viii, Bu^{t}OH, EDC, DMAP. EDC = 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, HOBt = 1-hydroxybenzotriazole, TBTU = *O*-(1*H*-benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

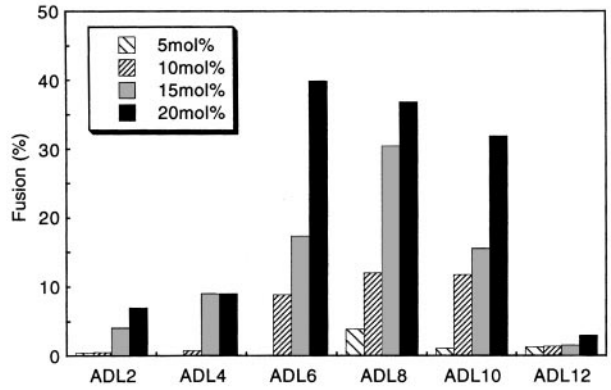


Fig. 1 Dependence of fusion % after 5 min reaction upon alkyl chain lengths of head groups of ADL*n*; pH 4.0, 37 °C; composition (mol%) of ADL*n* in liposomes is described inside the figure.

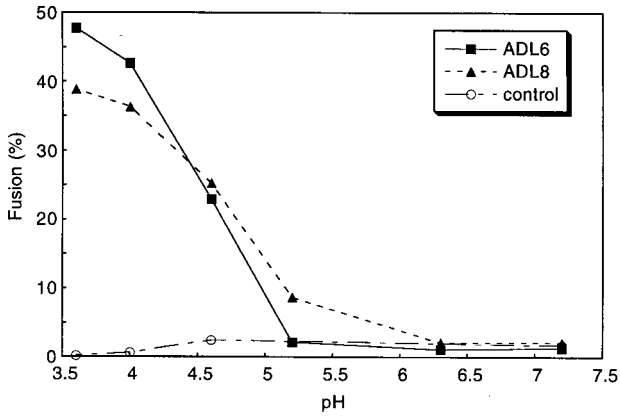


Fig. 2 pH profile of fusion % after 5 min reaction at 37 °C; [ADL*n*] = 20 mol%.

between ADL6 and ADL10. The reason for the very poor ability of ADL12 to induce liposome fusion may be that its alkyl chain in the head part is too long and too hydrophobic to remain in an outer aqueous phase and, consequently, ADL12 may be embedded in the membrane even at neutral pH. In contrast, the head alkyl chains of ADL2 and ADL4 are considered too short and too hydrophilic to migrate to another membrane, which may also result in low fusion %.

Notes and references

† All new lipids were fully characterized by ^{1}H NMR and mass spectroscopy and elemental analysis as follows. ADL2: δ_H(270 MHz, $CDCl_3$ and CD_3OD) 4.76 (t, 1H, CH, *J* 5.5), 4.62 (t, 1H, CH, *J* 5.9), 3.10–3.22 (m, 4H, 2NHCH_2), 2.78 (d, 2H, CH(COOH)CH_2, *J* 5.6), 2.46–2.73 (m, 6H, CHCH_2 and (CH_2)$_2$CO), 1.48 (br s, 4H, 2NHCH$_2$CH_2), 1.27 (br s, 52H, 2(CH_2)$_{13}$CH$_3$), 0.89 (t, 6H, 2CH2CH_3, *J* 6.4). Anal. Calcd for $C_{44}H_{82}N_4O_8$·0.7CF_3COOH: C, 62.32; H, 9.53; N, 6.40. Found: C, 62.00; H, 9.41; N, 6.16. FAB MS calcd: 795.6 (M + H$^+$). Found: 796. ADL4: δ_H(270 MHz, $CDCl_3$ and CD_3OD); 4.76 (t, 1H, CH, *J* 5.8), 4.64 (t, 1H, CH, *J* 6.1), 3.11–3.22 (m, 4H, 2NHCH_2), 2.79 (dd, 1H, CHCH_AH$_B$, *J* 5.9 and 15.7), 2.74 (dd, 1H, CHCH$_A$$H_B$, *J* 6.6 and 15.7), 2.64 (dd, 1H, CHCH_AH$_B$, *J* 5.9 and 15.2), 2.54 (dd, 1H, CHCH$_A$$H_B$, *J* 6.3 and 15.2), 2.33 (t, 2H, CH$_2$CH_2CO, *J* 6.9), 2.27 (t, 2H, CH2CH_2CO, *J* 7.3), 1.67 (m, 4H, 2CH_2CH$_2$CO), 1.48 (m, 4H, 2NHCH$_2$CH_2), 1.27 (br s, 52H, 2(CH_2)$_{13}$CH$_3$), 0.89 (t, 6H, 2CH$_2$CH_3, *J* 6.6). Anal. Calcd for $C_{44}H_{82}N_4O_8$·0.5CF_3COOH: C, 64.13; H, 9.91; N, 6.37. Found: C, 63.83; H, 9.83; N, 6.13%. FAB MS calcd: 823.7 (M + H$^+$). Found: 824. ADL6: δ_H(270 MHz, $CDCl_3$ and CD_3OD) 4.75 (t, 1H, CH, *J* 5.6), 4.64 (t, 1H, CH, *J* 6.1), 3.10–3.22 (m, 4H, 2NHCH_2), 2.78 (dd, 1H, CHCH_AH$_B$, *J* 5.8 and 15.3), 2.75 (dd, 1H, CHCH$_A$$H_B$, *J* 5.8 and 15.3), 2.63 (dd, 1H, CHCH_AH$_B$, *J* 5.9 and 15.0), 2.53 (dd, 1H, CHCH$_A$$H_B$, *J* 6.3 and 15.0), 2.30 (t, 2H, CH$_2$CH_2CO, *J* 7.4), 2.24 (t, 2H, CH$_2$CH_2CO, *J* 7.9), 1.62 (m, 4H, 2CH_2CH$_2$CO), 1.48 (m, 4H, 2NHCH$_2$CH_2), 1.33 (m, 4H, (CH_2)$_2$CH$_2$CH$_2$CO), 1.26 (br s, 52H, 2(CH_2)$_{13}$CH$_3$), 0.89 (t, 6H, 2CH$_2$CH_3, *J* 6.8). Anal. Calcd for $C_{48}H_{90}N_4O_8$: C, 67.73; H, 10.66; N, 6.58. Found: C, 67.60; H, 10.69; N, 6.40%. FAB MS calcd: 851.7 (M + H$^+$). Found: 852. ADL8: δ_H(270 MHz, $CDCl_3$ and CD_3OD) 4.76 (t, 1H, CH, *J* 5.6), 4.64 (t, 1H, CH, *J* 5.9), 3.10–3.22 (m, 4H, 2NHCH_2), 2.79 (dd, 1H, CHCH_AH$_B$, *J* 5.6 and 15.2), 2.75 (dd, 1H, CHCH$_A$$H_B$, *J* 5.6 and 15.2), 2.64 (dd, 1H, CHCH_AH$_B$, *J* 5.9 and 14.6), 2.53 (dd, 1H, CHCH$_A$$H_B$, *J* 6.3 and 14.6), 2.29 (t, 2H, CH$_2$CH_2CO, *J* 7.6), 2.23 (t, 2H, CH$_2$CH_2CO, *J* 8.3), 1.61 (m, 4H, 2CH_2CH$_2$CO), 1.48 (m, 4H, 2NHCH$_2$CH_2), 1.32 (m, 8H, (CH_2)$_4$CH$_2$CH$_2$CO), 1.26 (br s, 52H, 2(CH_2)$_{13}$CH$_3$), 0.88 (t, 6H, 2CH$_2$CH_3, *J* 6.6). Anal. Calcd for $C_{50}H_{94}N_4O_8$: C, 68.29; H, 10.78; N, 6.37. Found: C, 68.26; H, 10.78; N, 6.21%. FAB MS calcd: 879.7 (M + H$^+$), Found: 880. ADL10: δ_H(270 MHz, $CDCl_3$ and CD_3OD) 4.76 (t, 1H, CH, *J* 5.6), 4.63 (t, 1H, CH, *J* 5.9), 3.10–3.24 (m, 4H, 2NHCH_2), 2.74–2.84 (m, 2H, CHCH_2CO), 2.64 (dd, 1H, CHCH_AH$_B$, *J* 5.9 and 14.7), 2.52 (dd, 1H, CHCH$_A$$H_B$, *J* 5.9 and 14.7), 2.29 (t, 2H, CH$_2$CH_2CO, *J* 7.2), 2.23 (t, 2H, CH$_2$CH_2CO, *J* 8.6), 1.61 (m, 4H, 2CH_2CH$_2$CO), 1.48 (m, 4H, 2NHCH$_2$CH_2), 1.29 (br s, 12H, (CH_2)$_6$CH$_2$CH$_2$CH$_2$CO), 1.26 (br s, 52H, 2(CH_2)$_{13}$CH$_3$), 0.88 (t, 6H, 2CH$_2$CH$_3$, *J* 6.4). Anal. Calcd for $C_{52}H_{98}N_4O_8$·H$_2$O: C, 67.49; H, 10.89; N, 6.05. Found: C, 67.85; H, 10.77; N, 5.84%. FAB MS calcd: 907.7 (M + H$^+$), Found: 908. ADL12: δ_H(270 MHz, $CDCl_3$ and CD_3OD) 4.76 (t, 1H, CH, *J* 5.6), 4.63 (t, 1H, CH, *J* 6.1), 3.10–3.22 (m, 4H, 2NHCH_2), 2.70–2.84 (m, 2H, CHCH_2), 2.63 (dd, 1H, CHCH_AH$_B$, *J* 5.8 and 14.9), 2.52 (dd, 1H, CHCH$_A$$H_B$, *J* 6.3 and 14.9), 2.29 (t, 2H, CH$_2$CH_2CO, *J* 7.4), 2.23 (t, 2H, CH2CH_2CO, *J* 8.6), 1.62 (m, 4H, 2CH_2CH$_2$CO), 1.48 (m, 4H, 2NHCH$_2$CH_2), 1.26 (br s, 68H, (CH_2)$_8$CH$_2$CH$_2$CO and 2(CH_2)$_{13}$CH_3), 0.88 (t, 6H, 2CH$_2$CH_3, *J* 6.4). Anal. Calcd for $C_{54}H_{102}N_4O_8$·H$_2$O: C, 68.03; H, 10.99; N, 5.88. Found: C, 68.52; H, 10.83; N, 5.67%. FAB MS calcd: 935.8 (M + H$^+$), Found: 936.

‡ Egg PC was dissolved in $CHCl_3$–MeOH (3 : 1, v/v) with various amounts of ADL*n* for fusion liposome (Fusion-Lip), and with 0.5 mol% of NBD-PE and 0.5 mol% of Rh-PE for fluorescence-labeled liposome (Label-Lip). Lipid solutions were dried under a N_2 gas stream followed by the removal of residual solvent under high vacuum for 3 h. The resulting lipid films were hydrated by vortex-mixing with HEPES buffer (10 mM HEPES, 100 mM NaCl, pH 7.2) to make multilamellar vesicles (MLVs). MLVs were sonicated at 65 °C for 5 min by a probe-type sonicator and the resulting clear suspension (SUVs) was used in the experiments. After Fusion-Lip and Label-Lip (10:1, v/v) were mixed at 37 °C the lipid mixing assay was started by adding the necessary amount of citric acid (0.4 M) to adjust the pH.

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