New chemoenzymatic approach to glyco-lipopolymers: practical preparation of functionally active galactose–poly(ethylene glycol)–distearoylphosphatidic acid (Gal–PEG–DSPA) conjugate

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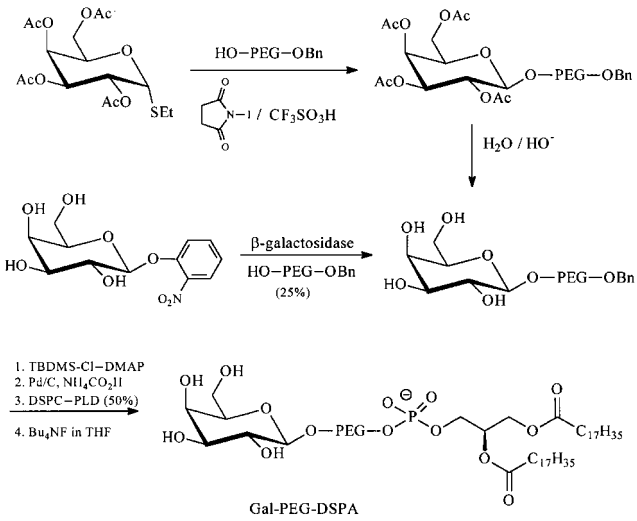
A practical approach to galactose–PEG–distearoylphosphatidic acid (DSPA) retaining full lectin binding, involves glycosylation of monobenzyl ether–PEG, suitable protection of the sugar hydroxy groups, debenzylation, followed by enzymatic transphosphatidylation with phosphatidylcholine and final deprotection.

The ability of poly(ethylene glycol) (PEG) conjugates of lipids, usually phosphatidylethanolamine (PE),[1] to increase systemic circulation and concomitantly decrease uptake of micelles or liposomes by the reticuloendothelial system is well recognized and routinely used. To combine this useful property with a ligand specific targeting, we and others introduced a series of ligand–PEG–lipid conjugates and their liposomes.[2] Among the ligands used in this capacity are antibodies, and fragments thereof, peptides, vitamins, and carbohydrates. The latter group of ligands is particularly promising. These are small and very polar molecules of low immunogenicity. There are a host of useful carbohydrate–receptor interactions suitable for utilization in PEG-grafted liposomes.[3] These ligands, even when individually of only weak binding affinity, maximize the targeting ability through a multivalent array presentation on the surface of a liposome. Saccharide–PEG–lipid conjugates are rather challenging synthetic targets. There are only a few examples of their preparation.[4–6] Here we describe a new chemoenzymatic approach to these constructs. The method is exemplified by the preparation of galactose–PEG–DSPA, which in its liposomal formulation exhibits substantial lectin-binding activity. This conjugate is potentially suitable for targeting liposomes to the asialoglycoprotein receptors expressed on liver hepatocytes.[7]

The synthetic pathways employed in this study are summarized in Scheme 1. Initially we explored preparation of PEG-yl–β-D-galactopyranoside (Gal–PEG). Enzymatic galactosylation of monobenzyl ether PEG (BnO–PEG–OH)[8] of molecular weight 2000 Da utilizing β-galactosidase proceeded well under modified conditions described by Matsushima *et al.*[9] resulting in formation of β-Gal*p*–O–PEG–OBn in 25% isolated yield. We also explored a two step chemical approach to the same product utilizing first iodonium ion promoted thioglycoside-mediated attachment of the peracetylated Gal*p* moiety,[10] followed by deacetylation. Although longer, this approach yielded galactosylated PEG derivatives in essentially quantitative yield. β-Glycosides were exclusively formed from both the enzymatic and chemical methods (Fig. 1a).

Recently we developed a new method for the preparation of phosphatidyl–PEG by an enzymatic process utilizing phospholipase D and distearoyl phosphatidylcholine.[11] To apply this reaction to Gal–O–PEG–OBn, the benzyl group had to be removed and the primary hydroxy group at C-6 of the galactose moiety had to be suitably protected.[12] Silylation with excess of TBDMS-Cl in presence of the DMAP provided the desired monoprotected saccharide along with several oversilylated products. Oversilylation had no bearing on the final outcome of the synthesis. Removal of the benzyl ether from the other terminal of the PEG chain proceeded quantitatively under catalytic transfer hydrogenation conditions with Pd/C and ammonium formate in MeOH.[13] Enzymatic elaboration of the distearoylphosphatidyl moiety proceeded smoothly in CCl_4–acetate buffer, pH 5.6, in 50% yield, and finally silyl groups



Scheme 1 Reaction pathways used in this study. Yields of the transformations shown were quantitative unless indicated in parentheses (PEG ≡ –(CH_2CH_2O)$_{44}$–CH_2CH_2–).

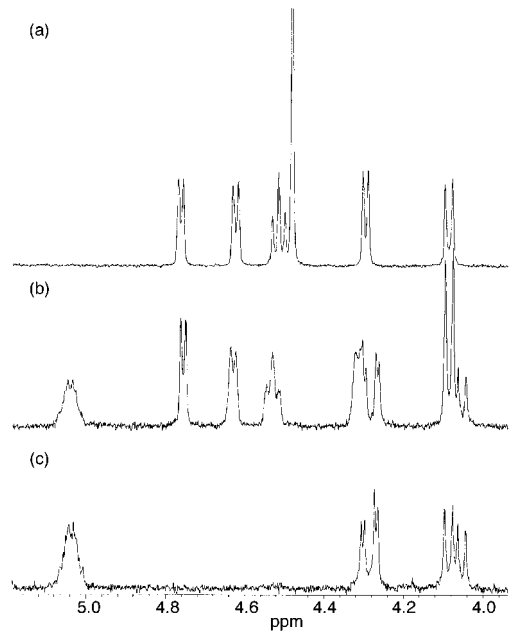


Fig. 1 ^{1}H-NMR (360 MHz, d_6-DMSO) spectra of (a) Gal–PEG–OBn, (b) Gal–PEG–DSPA, and (c) MeO–PEG–DSPA. Only the region 3.8–6 ppm containing the characteristic signals of the sugar and glycerophospho moieties is shown. Characteristic peaks of stearoyl (t 0.84, s 1.24, m 1.50, m 2.24 ppm in b and c only) and PEG (s 3.5 ppm) residues were present.

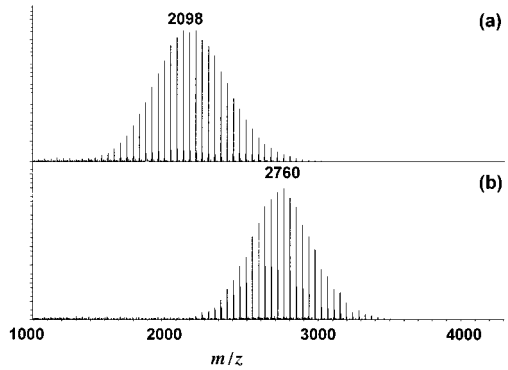


Fig. 2 MALDI-TOFMS of (a) Gal–PEG–OBn and (b) Gal–PEG–DSPA. Spectra were acquired on a PHI-EVANS MALDI triple electrostatic TOFMS analyzer using a 337 nm (600 ps pulse width) desorption laser, and gentisic acid as the matrix material.

were cleanly removed by fluoridolysis to provide Gal–PEG–DSPA. Fig. 1 illustrates the ^{1}H-NMR spectra of β-Gal*p*–O–PEG–OBn (a), β-Gal*p*–O-PEG–DSPA (b), and for comparison MeO–PEG–DSPA (c). The presence of a H-1 signal (d, 4.08 ppm, $J_{1,2}$ = 7.2 Hz) of the β-linked Gal residue as well as an additional primary and three secondary hydroxy groups is clearly seen. The final product also produced the characteristic signals of a glycerophospholipid moiety (dd at 4.1 and 4.3 ppm, as well as m at 5.1 ppm), the first two signals overlapping with the anomeric hydrogen and another secondary OH peak. Further corroboration of the structure of the glycosylated conjugates was obtained by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Fig. 2). Single-modal distributions of signals equally spaced at 44 Da intervals of the ethylene oxide repeating unit of PEG are clearly visible. Average molecular weights of the galactosylated PEG derivatives were in accordance with the expected values.

To ascertain the functional activity of Gal–PEG–DSPA, the conjugate was incorporated at 0, 1, and 5 mol% into egg-phosphatidylcholine large unilamellar vesicles. Interaction of the liposomes with *Ricinus communis* lectin (RCA-I) was monitored turbidometrically as illustrated in Fig. 3. It was observed that the lectin caused agglutination of the liposomes, which was more intense with a higher content of Gal–PEG–DSPA. The agglutination process could be inhibited by Gal–PEG–OBn; however, a 10-fold excess over liposome-bound Gal was required to achieve 50% inhibition. Complete inhibition was observed at 25-fold excess of the competitor. Similar experiments with lactose acting as a competitor showed that it was approximately 5-fold more effective at inhibiting agglutination than Gal–PEG–OBn (data not shown). Thus the multivalent liposome was binding more avidly than either one of the two monovalent competitors. It is interesting that the presence of MeO–PEG_{2000}–DSPA conjugate in the same lipid vesicles greatly inhibited agglutination. We previously observed a similar inhibitory effect on the binding of folate–PEG–liposomes to folate receptor-bearing cells.[14] It is pertinent to note that our findings do not support observations of Shimada *et al.*[5] They prepared Gal–PEG–lipids containing acyl glycosidic linkages and PEG tethers of different lengths, and reported that only liposomal preparations of the conjugates with decaethylene oxide spacer, and no longer, were agglutinated with RCA lectin.

We developed a practical method for the preparation of glyco-lipopolymers of the type Gal–PEG–DSPA. Lectin binding activity of the galactopyranoside moiety was well preserved, although it was inhibited if substantial amounts of MeO-PEG-lipid was included in the same liposomes. Monovalent

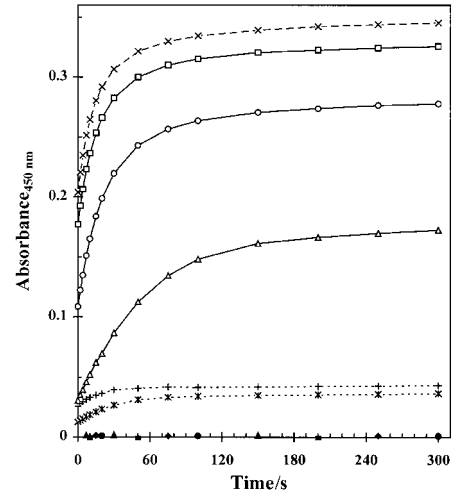


Fig. 3 Turbidometric measurements of RCA-I (50 μg ml^{-1}) mediated agglutination of Gal–PEG–DSPA containing liposomes (0.5 mM total lipid; 120–130 nm mean particle size). Liposomes containing 5 mol% Gal–PEG–DSPA (×), 5 mol% Gal–PEG–DSPA and 5 mol% MeO-PEG-DSPA (+), 1 mol% Gal–PEG–DSPA (✻), 1 mol% Gal–PEG–DSPA and 5 mol% MeO–PEG–DSPA (▲), without any PEG-lipids (●), 5 mol% MeO–PEG–DSPA (◆). Agglutination of liposomes containing 5 mol% Gal–PEG–DSPA in presence of Gal–PEG–OBn at 25 (□), 125 (○), 250 (△) and 625 μM (■).

Gal–PEG was more than one order of magnitude weaker at binding to the lectin than liposomal Gal–PEG–DSPA, suggesting that multivalent presentation of the carbohydrate moiety results in more effective binding.

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