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Synthesis of an Amphiphilic Tetraantennary Mannosyl Conjugate and Incorporation Into Liposome Carriers

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Abstract—We have synthesized a novel conjugate (Man_4K_3DOG) composed of a tetramannosyl head group connected, via a polyethylene glycol spacer, to a lipid moiety. This amphiphilic molecule was easily incorporated into the bilayers of liposomes. As expected from the clustering effect, such multivalent mannose residues when exposed on the surface of the vesicles showed much higher binding affinity for Concanavalin A than their monomannosyl analogue. Mannosylated liposomes prepared with the tetravalent antenna could be promising carriers for e.g., loading dendritic cells with antigens for vaccination purposes.

The human mannose receptor (MR) is predominantly present on macrophages and dendritic cells.[1] It binds carbohydrates present on the surface of microorganisms contributing to their clearance and takes part to the innate immunity.[2] The mannose receptor also strongly enhances effector immune functions through efficient antigen (ag) uptake and delivery to MHC class II molecules.[3] It has been demonstrated that the interactions of MR with carbohydrates are usually of low affinity unless these ligands are organized as multivalent clusters in specific arrangements.[4] In the search of effective MR ligands, several groups have synthesized multivalent glycopeptide mimics and demonstrated that the affinity of MR was enhanced for branched structures with valencies increasing from 2 up to 8 mannose residues.[5] Disparity of results about optimal valency was attributed to other factors affecting binding affinity and specificity, such as charge or ligand geometry.[6] Liposomes have been described as tools for the efficient presentation of ligands to cell surface receptors, which require multivalent contacts. Moreover, ag associated to such vesicles were also shown to be more efficiently captured and presented by antigen presenting cells than free ag in solution.[7]

Thus, in the present work, we have synthesized two novel neutral mannosyl amphiphilic compounds (Schemes 1 and 2). They have a hydrophilic head group, with one or four mannose residue(s), linked via a polyethyleneglycol spacer arm to a lipid moiety that allows their insertion into the bilayers of liposomes. Several considerations have guided our approach: (i) A tetramannosyl cluster was considered to represent a good compromise between its affinity for the human MR and synthetic accessibility;[5] (ii) These ligands when exposed at the surface of vesicles could in principle engage in multiple interactions, and thus dramatically increase their apparent affinity for the cells expressing MRs; (iii) The lengths of the spacer arms between the α-D-mannose groups and the scaffold, and between the ligand and the anchoring moiety, were chosen to provide a good flexibility and accessibility of the liposome-associated ligands to the MR carbohydrate recognition domains; (iv) The bilayer anchoring moiety contained oleyl chains to ensure to these lipids a low phase transition temperature and a good miscibility with the liposomal phospholipids. The feasibility of preparing liposomes with variable amounts of these neoglycolipids was assessed. The accessibility of the mannose residues on the surface of the vesicles and their ability to engage into multivalent interactions with the lectin concanavalin A (Con A) was also evaluated.

Synthesis of the Amphiphilic Tetramannosyl Conjugate Man_4K_3DOG[8]

The synthesis of **Man_4K_3DOG** (Scheme 1) first required the production of lipid **3**. Compound **1** was synthesized

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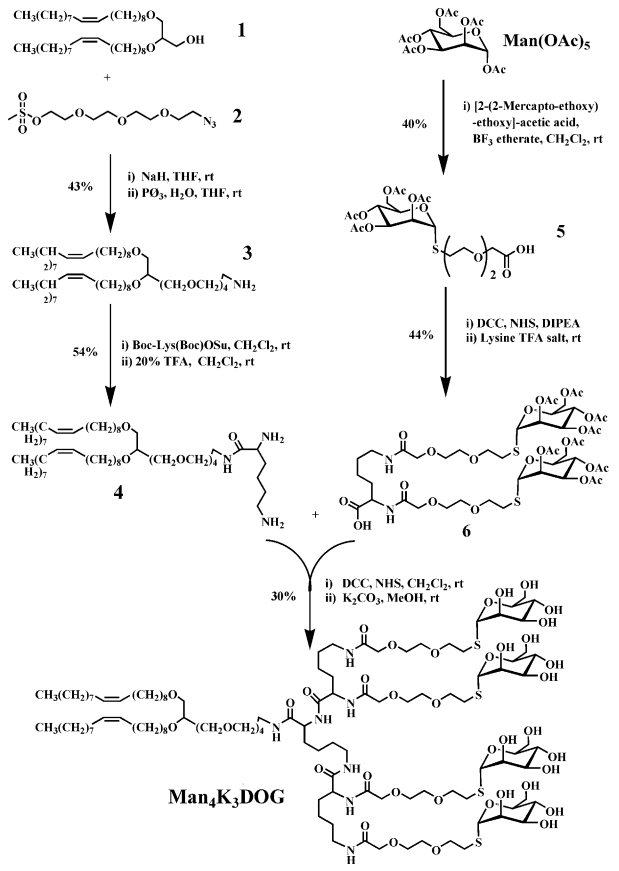


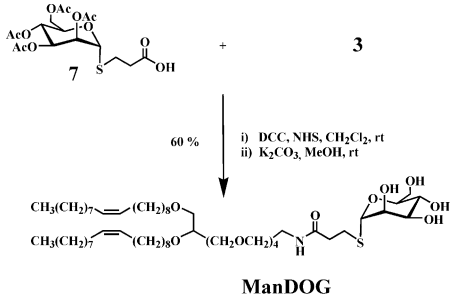
Scheme 1. Synthesis of the tetramannosyl conjugate **Man_4K_3DOG** (4 mannose residues attached to a tri-L-lysine scaffold that is conjugated to a dioleyl glycerol anchor).

in 4 steps starting from glycerol and oleyl alcohol with the same procedure as described previously.[9] Compound **2** was synthesized in 2 steps starting from tetraethylene glycol as described previously.[10] The primary alcohol of **1** was first alkylated with the methanesulfonyl ester **2**, in presence of NaH, to give the azido compound that was reduced with triphenylphosphine and water into the primary amine **3**. The free amine was reacted with the active hydroxysuccinimide ester Boc-Lys(Boc)-OSu to give the diprotected diaminolipid. Compound **4** was finally isolated, after cleavage of the protecting groups by a simple treatment with TFA. Compound **6** was obtained after introduction of two mannose groups on a L-lysine backbone. {2-(2-Mercapto-ethoxy)-ethoxy}-acetic acid, as described elsewhere,[11] was glycosylated with α-D-mannose pentaacetate in dichloromethane, catalyzed by boron trifluoride etherate,[12] to give **5**. The carboxylic acid function of **5** was activated with dicyclohexylcarbodiimide and *N*-hydroxysuccinimide at room temperature in the presence of diisopropylethylamine. L-lysine (TFA salt) in DMF was then added and the reaction was carried out for 12 h to give **6**. For the synthesis of **Man_4K_3DOG**, the carboxylic acid function of **6** was first converted into its *N*-hydroxysuccinimidyl ester. Compound **4** was then added and the reaction was carried out for 12 h. The final product **Man_4K_3DOG** was obtained after deprotection of the acetyl protecting groups of the mannose residues with potassium carbonate in methanol.

The monomannosyl conjugate **ManDOG** was also synthesized (Scheme 2) according to a similar procedure by reaction of compound **3** with **7**.[13]

Incorporation of ManDOG or Man_4K_3DOG into the bilayers of liposomes

Unilamellar liposomes were prepared with, phosphatidylcholine, phosphatidylglycerol, cholesterol (molar ratio 8:2:5) and variable amounts of the mono- or tetramannosyl lipids (Table 1).[14] The hydrodynamic diameter and zeta potential of the liposomes were determined by Photon Correlation Spectroscopy. As shown in Table 1, the incorporation of **ManDOG** did not change the mean diameter of these vesicles as compared to non-mannosylated liposomes (about 80–90 nm). Similarly, incorporation of **Man_4K_3DOG** scarcely affected the size of the vesicles that was increased to 100 nm. Importantly, the sugar residues associated to the vesicles, determined by the resorcinol-sulfuric acid



Scheme 2. Synthesis of the monomannosyl conjugate **ManDOG**.

Table 1. Characteristics of the mannosylated liposomes

	Expected mannose residues[a]	Measured liposomal mannose residues[b,c]	Mean size[c] (nm)	Zeta potential[c] (mV)
Blank liposomes	—	—	93.1±13.9	−32.3±1.4
ManDOG	1.41	1.34±0.3	86.7±0.8	−28.3±2.8
	2.85	2.83±0.3	79.6±11.1	−27.4±1.3
	4.24	4.52±0.7	85.7±11.7	−29.8±0.8
Man_4K_3DOG	1.41	1.16±0.8	101.1±1.2	−32.7±1.4
	2.84	2.90±0.8	107.3±2.0	−29.3±1.5
	4.26	3.63±1.1	116.9±2.9	−27.3±1.9

[a]Mannose residues incorporated into the liposome preparations (in mol % vs recovered liposomal phospholipids).[16]
[b]Mannose residues recovered in the liposomal preparations (in mol % vs recovered liposomal phospholipids).[16]
[c]Average values±S.D. ($n=3$).

Table 2. Parameters of liposomal **ManDOG** and **Man_4K_3DOG** aggregation by Con A. The results are expressed as rates of turbidity changes ($\Delta O.D./s \times 10^{-3}$) and concentrations (mM) of free mannose required to decrease the aggregation by 50% (IC_{50})

	Mannose content (mol % vs phospholipids)[a]	Rate of turbidity change	IC_{50} (mM)
ManDOG	1.34±0.3	0.38	2
	2.83±0.3	1.94	4
	4.52±0.7	2.11	5
Man_4K_3DOG	1.16±0.8	0.81	20
	2.90±0.8	3.10	30
	3.63±1.1	3.82	50

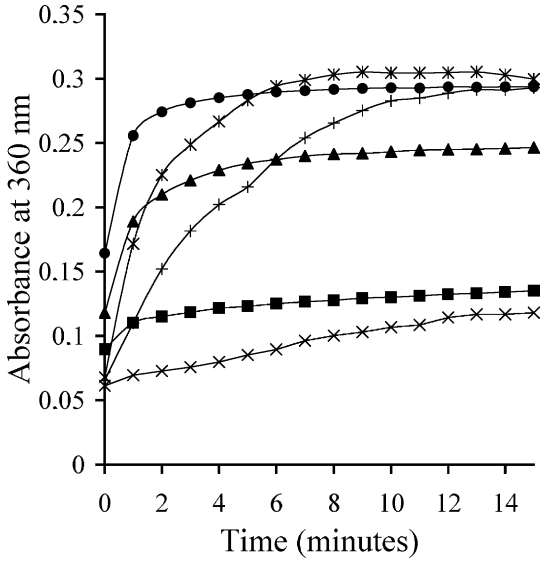


Figure 1. Absorbance changes at 360 nm of the liposome dispersions prepared with variable amounts of mono- or tetraantennary mannosyl conjugates at a Con A concentration of 0.125 mg/mL. [Liposome] = 60 μM phospholipids. ManDOG (mol% versus phospholipids): (X) 1.4; (+) 3; (✳) 4.8. Man_4K_3DOG (mannose mol % vs phospholipids): (■) 1.7; (▲) 3.2; (●) 4.9.

method,[15] increased proportionally with the amount of mannosyl lipids added during liposome preparation (Table 1); this indicates that our mannosyl amphiphilic conjugates were very efficiently inserted in the bilayers of vesicles. These mannosylated liposomes could also entrap in excellent yield solutes, such as 5(6)-carboxyfluorescein, and the resulting vesicles remained stable over weeks (S. Espuelas et al., unpublished).

Determination of the Accessibility of the Mannose Residues on the Surface of Liposomes

When the lectin ConA was added to suspensions of liposomes carrying mannose residues, the turbidity (measured at 360 nm) gradually increased and reached a plateau after about 15 min (Fig. 1). In contrast, no absorbance changes were observed with control liposomes (not shown). The subsequent addition of free α-D-mannopyranoside to the aggregates triggered a prompt and full decrease in turbidity, confirming that the change in absorbance was due to a specific recognition of the mannose residues on the surface of liposomes by ConA that resulted in an aggregation via multivalent interactions. The rates of turbidity changes and the free mannose concentrations required for 50% turbidity reduction (Table 2) increased with the surface density of the mannose residues. The aggregation was also dramatically influenced by the type of mannosylated lipid (mono-versus tetravalent) incorporated into the vesicles. For example in the case of liposomes containing 1.34 mol % **ManDOG** (Table 2), more than a 2000-fold molar excess of free mannose (IC_{50} about 2 mM) was needed to reduce the turbidity by 50%. This concentration was further increased 10-fold when a same mannose density at the surface of the liposomes was afforded by the tetramannosyl conjugate **Man_4K_3DOG**. We conclude that: (i) vesicles prepared with mono- or tetra-antennary mannosyl conjugates provide a multivalent arrangement that increases the affinity of these carbohydrates for Con A compared to free mannose residues, and (ii) the tetraantennary **Man_4K_3DOG**, inserted in the bilayers of liposomes, provides an important cluster effect and binding affinity for Con A.

In analogy to Con A, MR binds with higher affinity carbohydrate ligands organized as multivalent clusters. Moreover, it has been suggested that polyvalency in particular arrangements could amplify lectin-sugar binding specificity. Thus, our tetraantennary mannosylated liposomes could be promising carriers for drugs, genes and antigens that target cells expressing mannose receptors. The application of these liposomes for the targeting of human immature dendritic cells will be reported elsewhere.[17]

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8. Experimental procedures: All new compounds gave spectroscopic data in agreement with the assigned structures. ^{1}H NMR were recorded on a Bruker DPX 300 (300 MHz) spectrometer. Mass Spectra Analysis by Electro-Spray were performed using a Mariner ESI-Tof instrument from Applied Bio-System/Perking–Elmer. Conjugate **3**. Yield: 43%; ESI-TOF: Calculated: *m* 767.6160; *m*/*z* 768.6239 [M + H$^+$]. ^{1}H NMR δ (300 MHz, $CDCl_3$) 5.36–5.31 (m, 4H, C*H*=qC*H*), 3.83–3.39 (m, 23H, CH_2OCH_2, $CHOCH_2$), 3.08 (m, 2H, CH_2NH_2), 2.01–1.97 (m, 8H, $CH_2CH=CHCH_2$), 1.70 (m, 4H, $OCH_2CH_2CH_2$), 1.30 (m, 44H, CH_2 allyl), 0.87 (6H, t, $J=6.6$ Hz, CH_3). Conjugate **4**. Yield: 54%; ESI-TOF: Calculated: *m* 895,7953; *m*/*z* 896,7586 [M + H$^+$]. ^{1}H NMR δ (300 MHz, $CDCl_3$) 5.34–5.30 (m, 4H, C*H*=C*H*), 4.06 (m, 1H, NH_2CHCO), 3.63–3.37 (m, 25H, CH_2NH, CH_2OCH_2, $CHOCH_2$), 2.91 (m, 2H, CH_2NH_2), 2.01 (m, 8H, $CH_2CH=CHCH_2$), 1.74 (m, 4H, $CH_2CH_2CH_2NH_2$), 1.53 (m, 6H, NH_2CHCH_2, $OCH_2CH_2CH_2$), 1.30 (m, 44H, CH_2allyl), 0.88 (6H, t, $J=6.6$ Hz, CH_3). Conjugate **5**. Yield: 40%; ESI-TOF: Calculated: *m* 510.1407; *m*/*z* 533.1345 [M + Na$^+$]. ^{1}H NMR δ (300 MHz, $CDCl_3$) 5.36–5.24 (m, 5H, C*H* sugar), 4.39–4.11 (m, 4H, CH_2 sugar, OCH_2CO), 3.76–3.70 (m, 6H, $CH_2OCH_2CH_2OCH_2$), 2.93–2.74 (m, 2H, SCH_2), 2.18, 2.11, 2.06, 2.01 (s, 12H, CH_3CO). Conjugate **6**. Yield: 44%; ESI-TOF: Calculated: *m* 1130.3658; *m*/*z* 1154.2360 [M + H$^+$ + Na$^+$]. ^{1}H NMR δ (300 MHz, $CDCl_3$) 5.33–5.24 (m, 10H, C*H* sugar), 4.33–4.01 (m, 9H, CH_2 sugar, OCH_2CO, $NHCHCO$), 3.72–3.65 (m, 12H, $CH_2OCH_2CH_2OCH_2$), 3.31 (m, 2H, $NHCH_2CH_2$), 2.98–2.72 (m, 4H, SCH_2), 2.15, 2.08, 2.04, 1.98 (s, 24H, CH_3CO), 1.59–1.25 (m, 6H, $CHCH_2CH_2CH_2$). Conjugate **Man$_4$K$_3$DOG**. Yield: 30%; ESI-TOF: Calculated: *m* 2448.3458; *m*/*z* 2471.0760 [M + Na$^+$]. ^{1}H NMR δ (300 MHz, $CDCl_3$/ CD_3OD; 50/50) 5.33–5.30 (m, 8H, C*H*=C*H*, C*H*S), 4.40 (m, 3H, NHC*H*CO), 4.05–3.17 (m, 87H, CH_2OCH_2, C*H*OH, $CHCH_2OH$, $CHOCH_2$, CH_2NHCO) 2.93–2.74 (m, 8H, SCH_2), 2.03 (m, 8H, $CH_2CH=CHCH_2$), 1.70 (m, 4H, $CH_2CH_2CH_2O$), 1.59–1.25 (m, 18H, $CHCH_2CH_2CH_2$), 1.31 (m, 44H, CH_2 allyl), 0.87 (6H, t, $J=6.6$ Hz, CH_3). Conjugate **ManDOG**. Yield: 60%; ESI-TOF: Calculated: *m* 1017.7514; *m*/*z* 1040.7560 [M + Na$^+$]. ^{1}H NMR δ (300 MHz, $CDCl_3$/ CD_3OD; 50/50) 5.38–5.33 (m, 5H, CH=CH, C*H*S), 3.96–3.41 (m, 31H, CH_2OCH_2, C*H*OH, $CHCH_2OH$, CH_2OCH, CH_2NHCO) 2.99–2.55 (m, 4H, CH_2CH_2S), 2.03 (m, 8H, $CH_2CH=CHCH_2$), 1.70 (m, 4H, $CH_2CH_2CH_2O$), 1.31 (m, 44H, CH_2 allyl), 0.87 (6H, t, $J=6.6$ Hz, CH_3).

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14. **ManDOG** or **Man$_4$K$_3$DOG** were mixed with egg phosphatidylcholine, phosphatidylglycerol and cholesterol (80:20:50 molar ratio) in chloroform-methanol (9:1). The organic phase was evaporated to dryness under high vacuum and the lipid film was rehydrated in a 10 mM HEPES buffer containing 145 mM NaCl, pH 7.4. The obtained liposomal dispersion was further sonicated by an ultrasonifier (probe) for 1 h under a stream of nitrogen. The purification of the liposome dispersion was performed by gel filtration (Sephadex G-75). The phosphate content in the final preparation, estimated by the Rouser method, was found to be 85 ± 11% of the initially added phospholipids. The mannose contents of the liposomes (Tables 1 and 2) are given as mol % versus phospholipids, i.e., for a same mannose content, the vesicles were prepared with 4-times less **Man$_4$K$_3$DOG** than ManDOG.

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