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Recognition of Concanavalin A by cationic glucosylated liposomes

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ABSTRACT: The specificity of carbohydrate-lectin interaction has been reported as an attractive strategy for drug delivery in cancer therapy because of the high levels of lectins in several human malignancies. A novel cationic glucosylated amphiphile was therefore synthesized, as a model system, to attribute specificity toward D-glucose receptors to liposome formulations. Fluorescence experiments demonstrated that the monomeric glucosylated amphiphile is capable of interacting with fluorescently labeled Concanavalin A, a D-glucose specific plant lectin. The interaction of Concanavalin A with liposomes composed of a phospholipid and the glucosylated amphiphile was demonstrated by agglutination observed by optical density and dynamic laser light scattering measurements, thus paving the way to the preparation of other glycosilated amphiphiles differing for the length of polyoxyethylenic spacer, the sugar moieties and/or the length of the hydrophobic chain.

INTRODUCTION

The efficiency of existing and newly-developed therapeutics is often hampered by the absence of a proper control over biodistribution because they rely on non-targeted compounds. The possibility of directing a drug toward the diseased sites without changing its structure and hence its biological activity is fundamental for its therapeutic application. Encapsulation of drugs into targeted liposomes constitutes an optimum strategy for drug delivery because of the biocompatibility, low toxicity and easy of manufacturing of these lipid nanoparticles.[1] In fact the encapsulation of drugs into liposomes can improve their solubility in aqueous media, protect them, when necessary, from the action of lytic enzymes and facilitate their delivery inside the cells.[2] The specificity toward a given target can be obtained by decorating the liposome surface with proper homing devices, such as receptor ligands or antibodies, capable of controlling the biodistribution of the drug so that a large part of the dose reaches the target cells, thus reducing side effects.[3]

The functionalization of vesicles with natural or synthetic glycolipids might ascribe them specificity toward lectins, a class of non-enzymatic sugar-binding proteins whose most important function appears to be in cellular recognition and adhesion, including the infectivity of pathogens, immune response and reproduction.[4] Mannosylated liposomes, for example, were shown to preferentially target immune cells such as alveolar and peritoneal macrophages that overexpress a C-type lectin on their surface, attaining enhanced cellular uptake both *in vitro* and *in vivo*.[5-9] Glycoliposomes displaying galactose residues on their surface were effectively recognized by the galactose particle receptor (lectin) on the Kupffer cells.[10,11]

The specificity of carbohydrate-lectin interaction may indeed offer an attractive strategy for drug delivery in cancer therapy because the high levels of lectins such as galectin-3 in several human malignancies[12-15] can be exploited to convey glycosylated liposomes into these tumor cells.

Herein it is reported on the synthesis and the physicochemical characterization of the new glucosylated synthetic amphiphile **1** (Figure 1) and on the aggregates that it forms as a pure component and in formulation with dimyristoyl-*sn*-glycero-phosphocholine (DMPC). The binding of the monomeric glucosylated amphiphile with Concanavalin A (Con A), a soy bean lectin, was investigated by fluorescence experiments on Con A fluorescently labeled with fluoresceinisothiocyanate (FITC-Con A), whereas the binding of glucosylated liposomes DMPC/1 with the protein was investigated by agglutination monitored by optical density (OD) and dynamic laser light scattering (DLS) measurements.

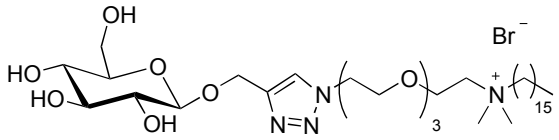

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Figure 1. Structure of the synthetic glucosylated amphiphile **1**.

EXPERIMENTAL SECTION

Instrumentation. NMR spectra were recorded on a Bruker 300 Avance spectrometer (operating at 300 for ^{1}H and 75 MHz for ^{13}C) and on a Bruker 400 Avance spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C). Deuterated solvents were used as internal standards.

HRMS-ESI spectrum was recorded on a LTQ Orbitrap XL instrument.

Steady-state fluorescence experiments were carried out on a Fluoromax-4 Horiba-Jobin Yvon spectrofluorimeter.

The optical density (OD) and UV measurements were carried out on Cary 300 UV–vis double beam spectrophotometer (Varian Australia PTY Ltd., Mulgrave, Vic., Australia).

Conductivity measurements were carried out on a Hanna conductimeter, HI-9932, equipped with a thermostating apparatus, in the temperature range 4–60 °C. All measurements were carried out in a jacketed cell that was maintained at the appropriate temperature (± 0.1 °C).

Dynamic light scattering (DLS) measurements were performed with a Malvern Nano-ZetaSizer, equipped with a 5 mW HeNe laser (wavelength = 632.8 nm) and a digital logarithmic correlator. The normalized intensity autocorrelation functions were measured at an angle of 173° at 25.0 ± 0.1 °C. The autocorrelation functions were analyzed by using the cumulant fit. The first cumulant was used to obtain the apparent diffusion coefficients D of the particles, further converted into apparent hydrodynamic diameters, d_h, by using the Stokes–Einstein relationship $d_h=k_BT/3\pi\eta D$, where k_BT is the thermal energy and η the solvent viscosity. The inverse Laplace transform (CONTIN) was also used to resolve correlation functions of multimodal system.

Materials. 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Fluoresceinisothiocyanate labeled Concanavalin A (FITC-Con A) was purchased from Invitrogen. Concanavalin A (Con A), from Canavalina ensiformis (Jack bean), glycogen, from bovine liver, PBS (Aldrich, 0.01 M phosphate buffer 0.0027 M KCl, 0.137 M NaCl, pH 7.4) and all reagents employed for the synthesis of **1** were purchased from Sigma-Aldrich.

*Preparation of compound **3**.* 20 mL of aqueous KOH 1M were added to a solution of 1.00 g of **2** (2.6 mmol) in 60 mL of EtOH. The mixture was stirred at room temperature for 1.5 hours and then neutralized by the addition of Amberlite IR-120. After filtration the solvent was removed under reduced pressure and the residue was purified by cromatography on silica gel (eluent CH2Cl2/MeOH=8/2) to give 0.33 g of 3 (yield 58%) as a white solid. 1H-NMR (δ CD3OD, 300 MHz) ppm: 2.86 (t, 4JHH = 2.88 Hz, C≡CH); 3.20 (t, 4JHH = 8.33 Hz, 1H, H-2); 3.26-3.41 (m, 3H, H-3, H-4, H-5); 3.63-3.69 (m, 1H, H-6); 3.84-3.89 (m, 1H, H-6); 4.41-4.45 (m, 1H , CH2C≡C); 4.48 (d, 3JHH = 8.33 Hz, 1H, H-1). ^{13}C-NMR (δ CD_3OD, 75 MHz) ppm: 55.2; 61.2; 70.0; 72.9; 74.2; 76.4; 76.5; 79.5; 100.6.

*Preparation of compound **4**.* 10 mL of aqueous NaOH 5 M and 45 mL of tetraethylene glycol (0.26 mol) were added to 10 mL of THF at 0 °C. A solution of 5.0 g of tosyl chloride (TsCl) (26 mmol) in 30 mL of THF was added dropwise at 0 °C and the reaction mixture was stirred until complete addition of TsCl. After diluting the mixture by adding 200 mL of THF, 800 mL of water and NaCl were added until complete phases separation. The organic layer was separated, the solvent was removed under reduced pressure and the residue dissolved in CH_2Cl_2. The organic solution was washed with water (10 x 150 mL) and dried over anhydrous Na_2SO_4. After filtration the solvent was removed under reduced pressure to afford 7.8 g of **4** (yield 85%) as a pale yellow oil. ^{1}H-NMR (δ $CDCl_3$, 300 MHz) ppm: 2.43 (s, 3H, CH_3); 2.65 (s, 1H, OH); 3.59-3.68 (m, 14H, CH_2OCH_2, $C\underline{H}_2OH$); 4.15 (t, $^3J_{HH}$ = 6.50 Hz, 2H, CH_2OS); 7.32-7.75 (m, 4H, CH). ^{13}C-NMR (δ $CDCl_3$, 75 MHz) ppm: 22.3; 61.7; 68.8; 69.3; 70.3; 70.4; 70.7; 70.8; 72.4; 128.0; 129.9; 138.2; 140.8.

*Preparation of compound **5**.* 1.98 g of **4** (5.7 mmol) and 0.50 g of NaN_3 (7.6 mmol) were suspended in 10 mL of dry DMF under nitrogen. The mixture was stirred and heated at 70 °C for 14 hours. The solvent was removed under high vacuum and the oily residue was dissolved in CH_2Cl_2. After filtration the solvent was removed under reduced pressure to afford 0.96 g of **5** (yield 77%) as a yellow oil. ^{1}H-NMR (δ $CDCl_3$, 300 MHz) ppm: 2.81 (s, 1H, OH); 3.38 (t, $^3J_{HH}$ = 6.02 Hz, 2H, CH_2N_3); 3.61-3.70 (m, 14H, CH_2O). ^{13}C-NMR (δ $CDCl_3$, 75 MHz) ppm: 50.7; 61.7; 70.0; 70.4; 70.6; 70.7; 72.5.

*Preparation of compound **6**.* 0.96 g of **5** (4.4 mmol) were dissolved in 15 mL of dry CH_2Cl_2 under nitrogen. The mixture was cooled to 0 °C and a solution of 0.13 mL of PBr_3 (1.5 mmol) in 10 mL of dry CH_2Cl_2 was added dropwise. A saturated aqueous solution of $NaHCO_3$ was added after two hours. The aqueous phase was washed with CH_2Cl_2 (2 x 20 mL), the organic layers were combined and dried over anhydrous Na_2SO_4. After filtration, the solvent was removed under reduced pressure and the oily residue was purified by chromatography on silica gel (eluent $CHCl_3$) to afford 0.49 g of **6** (yield 40%). ^{1}H-NMR (δ $CDCl_3$, 300 MHz) ppm: 3.37 (t, $^3J_{HH}$ = 6.04 Hz, 2H, CH_2N_3); 3.47 (t, $^3J_{HH}$ = 6.65 Hz, 2H, CH_2Br); 3.65-3.70 (m, 10H, OCH_2CH_2O, $C\underline{H}_2CH_2N_3$); 3.82 (t, $^3J_{HH}$= 6.65 Hz, 2H, $C\underline{H}_2CH_2Br$). ^{13}C-NMR (δ $CDCl_3$, 75 MHz) ppm: 30.3; 50.5; 69.9; 70.4; 70.5; 70.6; 71.0.

Preparation of compound 7. 0.32 g of **3** (1.5 mmol) and 0.42 g of **6** (1.5 mmol) were suspended in 6 mL of H_2O/*t*-BuOH (1:1). 150 µl of a freshly prepared aqueous solution of ascorbic acid 1 M and 50 µl of an aqueous solution of $CuSO_4 \cdot 5H_2O$ 0.3 M were then added to the mixture that was stirred at room temperature for 24 hours. The reaction mixture was then cooled to 0 °C and neutralized by an aqueous saturated solution of $NaHCO_3$; the solvent mixture was then removed under reduced pressure. The residue was dissolved in MeOH, and after filtration, the solvent was removed under reduced pressure. The residue was purified by cromatography on silica gel (eluent $CHCl_3$/MeOH=85/15) to give 0.64 g of **7** (yield 86%) as a pale yellow oil. ^{1}H-NMR (δ CD_3OD, 300 MHz) ppm: 3.20 (t, $^3J_{HH}$ = 8.69 Hz, 1H, H-2); 3.29-3.35 (m, 3H, H-3, H-4, H-5); 3.51 (t, $^3J_{HH}$ = 6.52 Hz, 2H, CH_2Br); 3.58-3.70 (m, 9H, H-6(1H), OCH_2CH_2O); 3.79 (t, $^3J_{HH}$ = 6.52 Hz, 2H,

$C\underline{H}_2CH_2Br$); 3.87-3.91 (m, 3H, H-6(1H), $NCH_2C\underline{H}_2$); 4.39 (d, $^3J_{HH}$ = 8.69 Hz, 1H, H-1); 4.53 (t, $^3J_{HH}$ = 6.25 Hz , 2H, NCH_2); 4.76-5.02 (m, 2H, $OCH_2C{=}C$); 8.15 (s, 1H, C=CH). ^{13}C-NMR (δ CD_3OD, 75 MHz) ppm: 30.2; 49.9; 61.5; 61.7; 69.0; 70.0; 70.1; 70.3; 71.0; 73.7; 76.6; 76.7; 102.2; 124.3; 144.1.

*Preparation of amphiphile **1**.* 0.65 mL (1.95 mmol) of N,N-dimethyl-N-hexadecylamine and 0.65 g of **7** (1.3 mmol) were suspended in 15 mL of CH_3CN. The mixture was heated to reflux for one week. The solvent was removed under reduced pressure and the residue was washed several times with Et_2O to give 0.62 g of the product **1** (yield 56%) as a pale yellow oil that resulted pure by elemental analysis. ^{1}H-NMR (δ CD_3OD, 400 MHz) ppm: 0.81 (t, $^3J_{HH}$ = 7.66 Hz, 3H, $CH_2C\underline{H}_3$); 1.22-1.42 (m, 26H, $N^+CH_2CH_2(C\underline{H}_2)_{13}CH_3$); 1.79 (m, 2H, $N^+CH_2C\underline{H}_2CH_2$); 3.18 (s, 6H, $CH_3N^+CH_3$); 3.21 (t, $^3J_{HH}$ = 7.96 Hz, 1H, H-2); 3.29-3.47 (m, 5H, H-3, H-4, H-5, $N^+C\underline{H}_2CH_2$ CH_2); 3.56-3.79 (m, 11H, H-6(1H), OCH_2CH_2O, $OCH_2C\underline{H}_2N^+$); 3.83-3.92 (m, 3H, H-6(1H), $C{=}CNCH_2C\underline{H}_2O$); 4.41 (d, $^3J_{HH}$ = 7.96 Hz, 1H, H-1); 4.59 (t, $^3J_{HH}$ = 6.38 Hz, 2H, $C{=}CNC\underline{H}_2CH_2O$); 4.76-5.00 m, 2H, OCH_2N); 8.11 (s, 1H, H-15). ^{13}C-NMR (δ CD_3OD, 100 MHz) ppm: 13.2; 21.7; 22.4; 22.5; 26.1; 28.9; 29.1; 29.2; 29.3; 29.4; 31.7; 50.1; 51.0; 61.3; 61.7; 63.1; 64.4; 65.6; 69.0; 70.0; 70.2; 70.3; 74.0; 76.6; 76.6; 102.2; 124.7; 144.1. HRMS: calculated for $C_{35}H_{69}N_4O_9$ $[M\text{-}Br^-]^+$: 689.5065; found: 689.5086 $[\alpha]_D$(CH_3OH, C= 0.81) = -10.35°. Elemental analysis: calc. for $C_{35}H_{69}N_4O_9 \cdot 5H_2O$: C, 48.89; H, 9.26; N, 6.52. Found: C, 49.01; H, 9.30; N, 6.49.

Methods. *Determination of the critical micellar concentration (cmc) of amphiphile **1** by conductivity.* Conductivity measurements were carried out at 25 °C by adding known volumes of a surfactant stock solution (0.176 M) to 30 mL of deionized water. Conductivity was measured after thorough mixing and temperature equilibration after each addition. The error in the conductivity measurements was within ± 0.1%. The plot of specific conductivity, *K*, versus the concentration of amphiphile **1** shows two linear trends whose intersection at $1.0 \cdot 10^{-3}$ M defines the *cmc*.[16,17]

*Binding of amphiphile **1** with FITC-Con A.* The affinity of amphiphile **1** with FITC-Con A was investigated at 25 °C by steady-state fluorescence experiments (at 525 nm; λ_{ex}= 488 nm) according to a reported procedure.[18] The fluorescence intensity I (corrected according to the dilution factor) of 2.5 mL of a solution containing 2.4 μg/mL of FITC-Con A and 3.2 μg/mL of glycogen in PBS was investigated as a function of the addition of small volumes of a

18.5 mM aqueous solution of amphiphile **1** in PBS. In a blank experiment the corrected fluorescence intensity I_B of 2.5 mL of a solution containing 2.4 μg/mL of FITC-Con A (in the absence of glycogen) was investigated as a function of the addition of small volumes of the aqueous solution of amphiphile **1** in PBS in order to obtain the same concentrations of amphiphile **1** of the previous experiment. The fluorescence increase percentage due to displacement of glycogen was thus evaluated by calculating ΔΔI(%) as defined in equation 1

$$\Delta\Delta I(\%)=\Delta I(\%)-\Delta I_B(\%)=[(I-I_0)/ I_0-(I_B -I_{B0})/ I_{B0}]100 \quad (1)$$

where I_0 and I_{B0} are the fluorescence intensities of the FITC-Con A/glycogen conjugate and of FITC-Con A, respectively, both in the absence of amphiphile **1**.

Liposome preparation. Aqueous dispersions of DMPC/**1** liposomes were prepared according to the procedure described by Hope et al.[19] Briefly, lipid films were prepared on the inside wall of a round-bottom flask by evaporation of solutions containing the proper amount of DMPC (dissolved in $CHCl_3$) and **1** (dissolved in MeOH) to obtain the desired molar percentage mixture. For experiments relative to Tm and surface potential determination of DMPC/**1** liposomes, the preparation of lipid films included Laurdan (dissolved in $CHCl_3$) and 4-heptadecyl-7-hydroxycoumarin (HC) (dissolved in THF), respectively.[21,22] The obtained lipid films were kept overnight under reduced pressure (0.4 mbar); then 3 mL of PBS were added to obtain a lipid dispersion of the desired concentration. The aqueous suspensions were vortex-mixed and then freeze-thawed six times from liquid nitrogen to 40 °C. Lipid suspensions were then extruded (10 times) through a 100 nm polycarbonate membrane.

Determination of aggregate size by DLS. A solution of amphiphile **1** above *cmc* (50 mM) in filtered bidistilled water and suspensions of DMPC/**1** (95:5) and DMPC/**1** (90:10) liposomes (1.25 mM total lipids in PBS) were analyzed by DLS measurements soon after preparation and after 48 hours.

Tm determination by OD measurements. Thermal phase transition of mixed liposomes was determined from the temperature-dependent changes of optical density (OD) at 260 nm.[20]

Measurements were made at 2.5 mM total lipid concentration in PBS. The temperature scan, in the 20-35 °C range, was carried out a 1 °C/min rate. Results were expressed in turbidity (OD) as a function of temperature; in the obtained plot the Tm of the liposome formulation corresponds to the inflection point.

Tm determination by fluorescence experiments. Tm was also determined by an indirect method described in literature that exploits Laurdan as a fluorescent probe.[21] Emission spectra (λ_{ex}= 360 nm) of liposome suspension (1 mM total concentration) of DMPC/**1**/Laurdan (95:5:0.1) in PBS were acquired as a function of temperature in the range 20-35 °C. Temperature was controlled by a circulating bath and the actual temperature was measured in the sample cuvette by a thermocouple. The generalized polarization parameter (GP) was calculated by equation 2

$$GP = (I_B - I_R)/(I_B + I_B) \quad (2)$$

where I_B (450 nm) and I_R (490 nm) are the fluorescence emission intensities corresponding to the emission maxima of Laurdan in the gel and in the liquid crystalline phase, respectively. The transition temperature corresponds to the inflection point in the GP versus temperature plot.

*Determination of surface potential (ψ°) of DMPC/**1** (95:5) liposomes.* Surface potential was determined by an indirect method described in the literature that exploits the pH-sensitive fluorophore HC.[22] Fluorescence measurements of liposome suspension (5 mM total concentration) of DMPC/1/HC (95:5:0.3) in PBS were performed by scanning the excitation wavelength between 300 and 400 nm, at an emission wavelength of 450 nm, varying the pH of solution between 2 and 12 by addition of concentrated aqueous sodium hydroxide or hydrochloric acid. The extent of dissociation of liposome included HC was monitored by the ratio of the excitation fluorescence intensities at 380 nm and 330 nm (pH independent isosbestic point). pK_a of HC associated with the cationic liposome bilayer ($pK_a^{charged}$) corresponds to the inflection point of the plot of I_{380}/I_{330} ratio

as a function of pH. The surface potential (ψ°) was obtained by a conversion and rearrangement of Boltzmann equation 3

$$\psi^\circ = -e^{-1}(pK_a^{charged} - pK_a^{neutral})k_B T \ln 10 \quad (3)$$

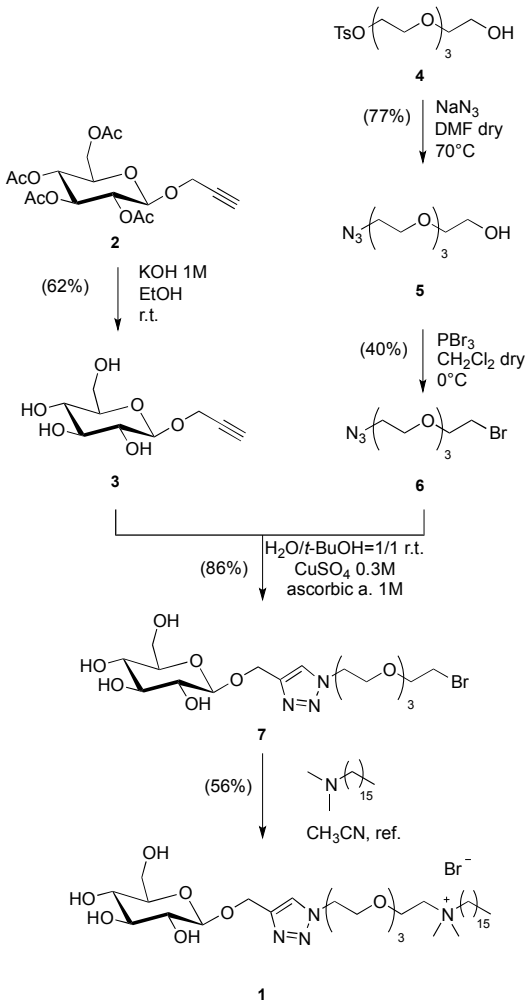
where k_B is the Boltzmann constant, T is the absolute temperature, *e* is the electron charge and and $pK_a^{neutral}$ is the pK_a of HC associated with neutral lipid bilayers (DMPC).

Agglutination of glucosylated liposomes by Con A investigated by OD measurements. The agglutination of DMPC/**1** (95:5) liposomes in the presence of Con A was determined from the time-dependent changes of specific turbidity of a 3 mL sample of 0.83 mM DMPC/**1** (95:5) liposomes in PBS in a 1-cm quartz cell upon addition of Con A (0.33 mg/mL final concentration) in PBS. Scans were carried out at 525 nm immediately after mixing and every minute for 200 minutes.

Agglutination of glucosylated liposomes by Con A investigated by DLS measurements. The agglutination of functionalized liposomes in the presence of Con A in the same experimental conditions of OD measurements, described above, was determined from the time-dependent change of the apparent hydrodynamic diameter of particles in suspension, evaluated by DLS measurements. Scans were carried immediately after mixing and at selected times in the time range of 2 hours.

RESULTS AND DISCUSSION

Synthesis and characterization of amphiphile 1. The amphiphilic properties of **1** result from the presence of a hexadecyl hydrophobic chain linked to a polar portion, *i.e*. a quaternary charged nitrogen linked to a glucose moiety (*i.e.* the glucose receptor recognition functionality) by a hydrophilic spacer that should guarantee a good exposure of the sugar on the liposome surface. Amphiphile **1** was synthesized according to the synthetic pathway reported in Scheme 1.



Scheme 1. Synthesis of glucosylated amphiphile **1**.

The key step of the convergent synthesis is the Cu(I)-catalyzed "click" reaction between the azido-compound **6** and the terminal alkyne **3** to obtain, in high yield and under mild reaction conditions, the bromo-intermediate **7** containing the glucose moiety and the hydrophilic spacer. The click adduct **7**, confirmed by the presence of the triazolic signal at 8.15 ppm in the ^{1}H NMR spectrum, was obtained in relative high yield (86%), though lower than yields generally obtained in click reactions, probably because the presence of the glucose moiety involves a loss of product in the purification on silica gel. The 1,4-disubstitution of the triazolic ring was confirmed by the presence of the correlation peaks between the signal due to the triazolic proton and the two signals due to the methylenic protons at 4.53 and 4.76-5.02 ppm in the ^{1}H-^{1}H NMR NOESY spectrum.

The azido-compound **6** was obtained starting from tetraethylene glycol by simple synthetic steps only one of which, *i.e.* the bromination of **5** to obtain **6**, required purification by chromatography on silica gel. Alkyne **3** was obtained by deacetylation of commercially available glucopyranoside **2** under alkaline conditions. Amphiphile **1** was finally obtained by alkylation of N,N-dimethylhexadecylamine with compund **7**.

Krafft point and Krafft temperature of **1** were evaluated as <4 °C because the 20 mM aqueous solution of **1** is completely transparent and stable at 4 °C.

The *cmc* of amphiphile **1** was determined by conductivity measurements in deionized water at 25°C, and was found to be $1.0 \cdot 10^{-3}$ M.

The DLS analysis of a solution of amphiphile **1** above its *cmc* (50 mM in deionized water) at 25°C indicated the presence of micelles characterized by hydrodynamic diameter of about 6 nm.

Interaction of monomeric amphiphile 1 with Con A. The binding affinity of amphiphile **1** with Con A was investigated by fluorescence experiments exploiting fluorescently labeled Con A, FITC-Con A. The fluorescence of FITC-Con A is quenched upon complexation with multibranched polysaccharides of D-glucose such as glycogen and is restored by addition of monomeric D-glucose.[18] It was thus expected that glucosylatedamphiphile **1** could displace glycogen from the binding sites of FITC-Con A, thus dequenching its fluorescence, similarly to D-glucose. Therefore, we investigated the fluorescence emission spectrum of a FITC-Con A/glycogen conjugate solution upon the addition of amphiphile **1**. As expected, the addition of **1** to a PBS solution containing the FITC-Con A/glycogen conjugate restored the fluorescence of the protein as shown in Figure 2. This indicates that the glucose moiety of amphiphile **1** is able to displace glycogen and to bind with Con A.

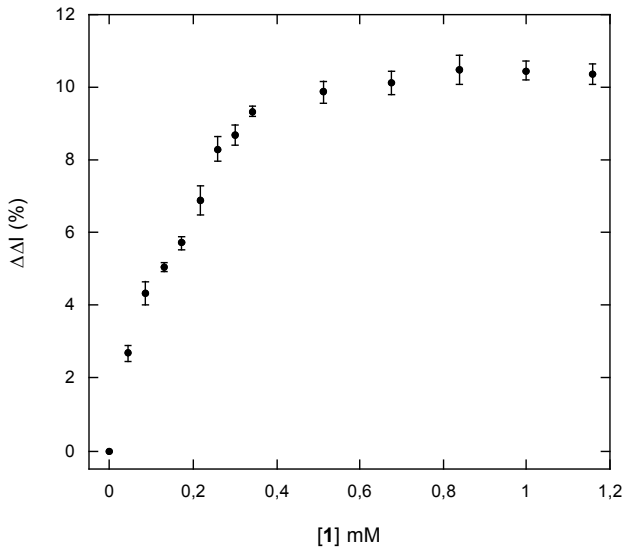


Figure 2. Effect of the addition of an aqueous solution of amphiphile **1** on the fluorescence intensity of a solution of FITC/Con A/glycogen conjugate in PBS. FITC/Con A initial concentration 2.4 μg/mL; glycogen initial concentration 3.2 μg/mL. The given values are calculated as the mean and standard deviation of three samples.

Preparation and characterization of mixed liposomes DMPC/1. Mixed DMPC/**1** liposomes were prepared at 95:5 and 90:10 lipid ratios. The DLS analysis, performed soon after extrusion and after 48 hours, indicated the presence of a stable monomodal distribution of liposomes characterized by a hydrodynamic diameter of ~120 nm only in the case of the 95:5 formulation, whereas a multimodal distribution was found in the case of the 90:10 formulation. Thus further investigations were carried out only on the 95:5 DMPC/**1** formulation.

Transition Temperature. The transition temperature of the 95:5 DMPC/**1** liposome formulation was measured by both turbidimetric and fluorescence experiments. Both procedures indicated that the presence of 5% of **1** in the formulation does not induce any significant change in the organization of the lipid bilayer with respect to DMPC. In fact the values of Tm obtained by turbidimetric and fluorescence method were 24.2 °C and 24.5 °C (Figure 3), respectively (the Tm of DMPC being 24.1 °C).

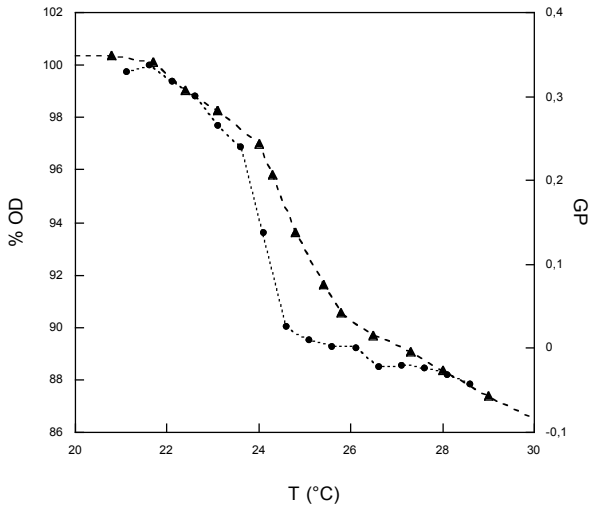


Figure 3. Percentage variation of optical density OD (•) at 260 nm reported as percentage, and generalized polarization parameter, GP (▲), of a DMPC/**1** (95:5) liposome suspension (1 mM and 5 mM in total lipids for OD and GP experiment, respectively) plotted as a function of temperature. The inflection points define the Tm. The given values are calculated as the mean of three samples.

Surface potential. The surface potential (ψ°) of DMPC/**1** (95:5) liposomes was measured according to a described procedure that exploits the spectrofluorimetric determination of the K_a of HC at the surface of liposomes as shown in Figure 4 and calculates the ψ° by including the pK_a value in eq. 3.[22] DMPC/**1** (95:5) liposomes are characterized, as expected, by a positive value of potential surface ψ° (23 ± 3 mV).

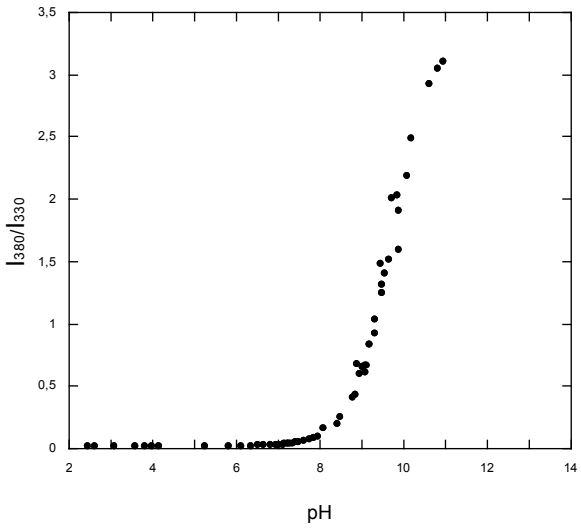


Figure 4. Titration curve relative to HC included in DPPC/**1** (95:5) liposomes; the inflection point defines the $pK_a^{charged}$. The given values are calculated as the mean of three samples.

Interaction of DMPC/1 (95:5) liposomes with Con A. Next we investigated if the inclusion of **1** in the DMPC lipid bilayer actually attributes it specificity toward a glucose receptor. Con A is a tetrameric protein with four binding sites for glucose. Because of the presence of multiple binding sites, the interaction of Con A with glucose decorated vesicles should result in the aggregation of vesicles, known as agglutination. Agglutination involves an increase of the dimension of particles in solution that can be detected by OD and DLS measurements.

The increment of OD of the liposome suspension observed upon the addition of Con A indicated the increase of particle size due to agglutination of vesicles; further, after ~260 minutes a strong decrease of OD, due to particle precipitation, was observed (Figure 5). In the absence of **1**, the agglutination of DMPC liposomes in the presence of Con A also occurred, due to non specific interaction of the protein with the DMPC lipid bilayer, however it occurred over a longer length of time with respect to glucosylated liposomes. Actually in this case (DMPC liposomes devoid of **1**) OD changes were not detected whithin 4 hours and precipitation was observed only after 24 hours. In the absence of Con A, neither OD increase nor precipitation was observed.

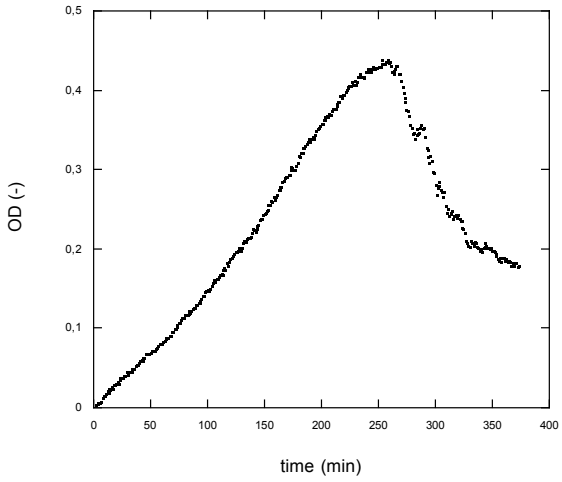


Figure 5. Effect of the addition of Con A on the optical density, OD, of a suspension of 95:5 DMPC/**1** liposomes in PBS, over time. OD increases due to the agglutination process; after ~260 minutes liposome precipitation involves a decrease of OD. Con A concentration 0.33 mg/mL; total lipids concentration 0.83 mM. Similar results are obtained replicating the experiment on three different samples.

The results obtained by OD were confirmed by DLS analysis of the agglutination experiment carried out in the same conditions. In fact the apparent hydrodynamic diameter increased within four hours, from 120 nm to 180 nm, upon the addition of Con A to the liposome suspension of DMPC/**1** (95:5) (Figure 6). Also in this case after ~260 minutes the precipitation of vesicles was observed.

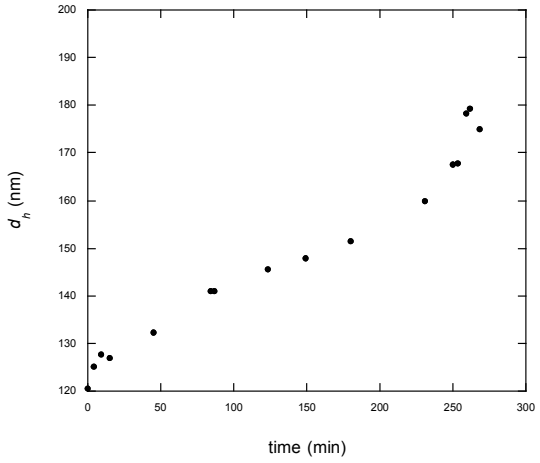


Figure 6. Effect of the addition of Con A on the apparent hydrodynamic diameter (d_h) of a suspension of DMPC/**1** (95:5) liposomes in PBS. Con A concentration 0.33 mg/mL; total lipids concentration 0.83 mM. Similar results are obtained replicating the experiment on three different samples.

CONCLUSIONS

We synthesized a novel glucosylated amphiphile by a synthetic strategy that exploits a "click chemistry" reaction. The results of the investigation indicated that the glucosylated amphiphile is capable of interacting specifically with Con A both as a monomer and when included in DMPC liposomes. The designed molecular backbone is therefore suitable for the development of glycosilated liposomes and an analogous synthetic pattern can be applied to other carbohydrates such as galactose or lactose to obtain lipid components specific toward human lectins.

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