Accepted Manuscript

Structurally related glucosylated liposomes: Correlation of physicochemical and biological features

Alessandro Mauceri, Luisa Giansanti, Giuseppina Bozzuto, Maria Condello, Agnese Molinari, Luciano Galantini, Antonella Piozzi, Giovanna Mancini

PII: S0005-2736(19)30135-X
DOI: https://doi.org/10.1016/j.bbamem.2019.06.003
Reference: BBAMEM 83004

To appear in: *BBA - Biomembranes*

Received date: 25 January 2019
Revised date: 29 May 2019
Accepted date: 7 June 2019

Please cite this article as: A. Mauceri, L. Giansanti, G. Bozzuto, et al., Structurally related glucosylated liposomes: Correlation of physicochemical and biological features, BBA - Biomembranes, https://doi.org/10.1016/j.bbamem.2019.06.003

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Structurally related glucosylated liposomes: correlation of physicochemical and biological features

*Alessandro Mauceri,[§] Luisa Giansanti,[#‡] Giuseppina Bozzuto,[†] Maria Condello,[†] Agnese Molinari,[†] Luciano Galantini,[§] Antonella Piozzi[§] and Giovanna Mancini[#]**

[§]Dipartimento di Chimica Università degli Studi di Roma "Sapienza", P.le A. Moro 5, 00185 Roma (Italy). [‡]Dipartimento di Scienze Fisiche e Chimiche, Università degli Studi dell'Aquila, Via Vetoio, 67010 Coppito, Italy. [#] CNR–Istituto per i Sistemi Biologici, Via Salaria km 29.300, 00016 Monterotondo Scalo (RM), Italy. [†]Centro Nazionale per la Ricerca e la Valutazione preclinica e clinica dei Farmaci, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy.

KEYWORDS: lectins, agglutination, glucosylated liposomes, active drug delivery, human breast cancer cells.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

ABSTRACT

Liposomes functionalized on their surface with carbohydrates (glycoliposomes) represent an optimal approach for targeting of drugs to diseased tissues *in vivo*, thanks to biocompatibility, low toxicity and easy manufacturing of these lipid nanoparticles. Here we report on the study of liposomes including a novel glycosylated amphiphile and on the comparison of their features with those of glycosylated analogues described previously. Further, the capability of the different glucosylated formulations to interact with three breast cancer cell lines was investigated. Our results show that the hydrophobic portion of the lipid bilayer strongly influences both the properties and the internalization of glycosylated liposomes.

INTRODUCTION

Glycolipids are amphiphilic molecules contains a saccharidic moiety as hydrophilic headgroup. They are in general biocompatible, show good solubility and are largely investigated as potential components of thermotropic and lyotropic liquid crystals, pharmaceutical products, or lubricants. Many biological processes involving cell–cell interactions such as signaling, recognition and adhesion are initiated, maintained and supported by carbohydrate–protein interactions and are modulated by glycans or glycolipids located on cell surface.[1] Therefore, glycolipids are considered bioadhesives and have been included in many potential drug delivery systems such as niosomes,[2] liposomes and lipid nanoparticles[3-5] to increase their specificity towards specific target cells and tissues. Galactosylated and mannosylated liposomes are largely investigated for targeting drug delivery; the former were used as selective targeting agents *via* specific receptor-mediated uptake in cancer cells,[6-9] whereas the latter were studied for

ACCEPTED MANUSCRIPT

macrophage and/or dendritic cells selective targeting via mannose receptor-mediated uptake in various tissues.[10-14]

The functionalization of liposomes with glycosylated amphiphiles might increase their specificity toward lectins, a class of non-enzymatic proteins, mostly located on cell membrane, that recognize and bind selectively with carbohydrates. The high specificity of carbohydrate–lectin interactions has already been studied as a strategy for drug delivery by using either carbohydrates or lectins as homing devices to target specific diseased cells or tissues. For example, the elevated levels of some lectins, such as galectin-3, on the surface of metastatic murine and human cancer cells suggest the attractive possibility to exploit these proteins as targets of glycosylated carriers in anticancer therapies.[15-18] A huge number of synthetic glycosylated ligands differing for the saccharidic residues and the lipophilic segment have been prepared and explored,[19,20] In fact many parameters such as the configuration and the conformation of the carbohydrate moiety and its proper exposure on the surface of the lipid nanocarrier, together with water molecules bound on liposome surface, counterions and nanoparticle dynamics control the recognition process.[21]

We previously demonstrated by agglutination experiments the capability of liposomes composed of 1,2-dimistroyl-*sn*-glycero-phosphocholine (DMPC) and any of the two synthetic glucosylated amphiphiles, **1** and **2** (Chart 1) to interact with plant lectin Concanavalin A (Con A).[22,23]

Herein we report on the synthesis and physicochemical characterization of the new glucosylated amphiphile **3** (Chart 1) that, due to the presence of two aliphatic chains in the hydrophobic portion, interacts differently with liposome membranes with respect to amphiphiles **1** and **2** and, most importantly, exerts a minor detergent effect thus allowing being included in

ACCEPTED MANUSCRIPT

larger amounts in liposome formulations. Liposomes composed of amphiphile **3**, as single component and in formulation with DMPC at different molar ratios, were investigated. The binding of **3** was investigated by the displacement of glycogen from Con A fluorescently labeled with fluorescein-isothiocyanate (FITC-Con A); displacement experiments were carried out on **3** as a monomer (*i.e.* as a pure component, below its cmc) and when included in DMPC liposomes, by fluorescence measurements, and by agglutination experiments, respectively. Furthermore, cell uptake of glucosylated liposomes DMPC/**1**, DMPC/**2** and DMPC/**3,** labeled with fluorescent 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE), was evaluated by laser scanning confocal microscopy and by flow cytometry on three human breast cancer cell lines (MCF7, MDA-MB-231 and SKBR3) expressing a glucose receptor.

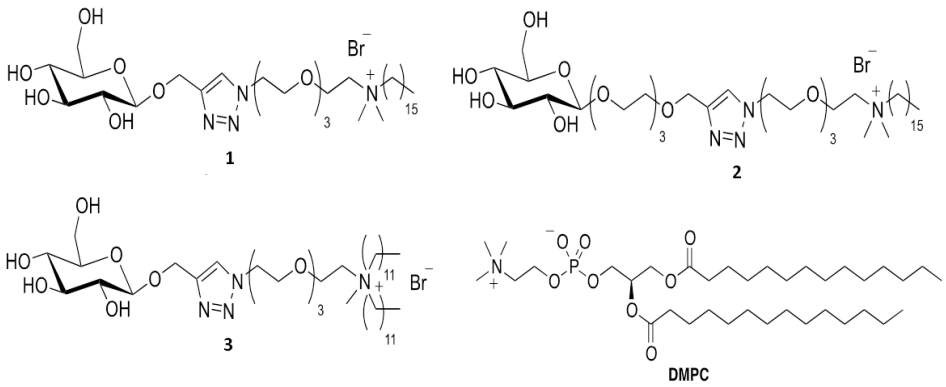


Chart 1. Liposome components.

METHODS

Instrumentation

ACCEPTED MANUSCRIPT

A Bruker 300 Avance spectrometer (operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C) and a Bruker 400 Avance spectrometer (operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C) were used to record NMR spectra. ^{1}H resonances of deuterated solvents were used as internal standards.

A LTQ Orbitrap XL instrument was used to record HRMS-ESI spectrum.

A Fluoromax-4 Horiba-JobinYvon spectrofluorimeter was used to carry out steady-state fluorescence experiments.

A Cary 300 UV–vis double beam spectrophotometer (Varian Australia PTY Ltd., Mulgrave, Vic., Australia) was used to carry out OD and UV measurements.

A Malvern Nano-ZetaSizer spectrometer, equipped with a 5 mW HeNe laser (λ_{exc} = 632.8 nm) and a digital logarithmic correlator was used to perform DLS measurements. 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 is the solvent viscosity.

A METTLER TA 3000 calorimeter provided with a TC 10 A processor by keeping the cell (DSC30) under N_2 flow was used to perform DSC measurements.

Materials

DMPC and NBD-PE were purchased from Avanti Polar Lipids (Alabaster, AL, USA). FITC-Con A was purchased from Invitrogen. Laurdan (6-Dodecanoyl-N,N-dimethyl-2-naphthylamine) and 4-heptadecyl-7-hydroxycoumarin (HC) were purchased from Fluka. Con A, from *Canavalia ensiformis* (Jack bean), glycogen, from bovine liver, phosphate-buffered saline (PBS; Aldrich; 0.01 M phosphate buffer; 0.0027 M KCl; 0.137 M NaCl; pH 7.4), and all reagents employed for

ACCEPTED MANUSCRIPT

the synthesis of amphiphile **3** were purchased from Sigma-Aldrich. Amphiphiles **1** and **2** were prepared as previously described.[22,23]

Synthetic procedures to prepare amphiphile 3

Preparation of N,N-didodecylmethylamine. 3.6 mL (14 mmol) of N-dodecylmethylamine and 6.4 mL (27 mmol) of 1-bromododecane were dissolved in 30 mL of absolute EtOH. The mixture was heated to reflux for two days. The solvent was removed under reduced pressure, the residue was dissolved in $CHCl_3$ and washed three times with a saturated aqueous solution of $NaHCO_3$. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. The oily residue was purified by chromatography on silica gel (eluent $CHCl_3$) to afford 2.89 g of *N*,*N*-didodecylmethylamine (yield 55%). ^{1}H-NMR (δ $CDCl_3$, 300 MHz) ppm: 0.84 (t, $^3J_{HH}$ = 9.18 Hz, 6H, $CH_2C\underline{H}_3$); 1.16-1.29 (m, 36H, $NCH_2CH_2(C\underline{H}_2)_9CH_3$); 1.54 (m, 4H, $NCH_2C\underline{H}_2$); 2.34 (s, 3H, NCH_3); 2.49 (m, 4H, NCH_2). ^{13}C-NMR (δ $CDCl_3$, 75 MHz) ppm: 14.11; 22.69; 27.28; 27.63; 29.36; 29.64; 31.92; 42.31; 57.91.

*Preparation of amphiphile **3***. (Scheme 1) 300 mg (0.60 mmol) of **4** (prepared as described previously)[22] and 239 mg (0.65 mmol) of *N*,*N*-didodecylmethylamine were suspended in 5 mL of anhydrous CH_3CN. The mixture was heated to reflux for one week. The solvent was removed under reduced pressure and the oily residue was purified by chromatography on silica gel (eluent from $CHCl_3$ 100% to $CHCl_3$/MeOH 80:20) to afford 0.62 g of the product **3** (yield 56%) as a pale yellow oil. ^{1}H-NMR (δ CD_3OD, 400 MHz) ppm: 0.85 (t, $^3J_{HH}$ = 7.88 Hz, 6H, $CH_2C\underline{H}_3$); 1.20-1.40 (m, 36H, $N^+CH_2CH_2(C\underline{H}_2)_9CH_3$); 1.71 (m, 4H, $N^+CH_2C\underline{H}_2CH_2$); 3.06 (s, 3H, N^+CH_3); 3.19 (t, $^3J_{HH}$ = 7.96 Hz, 1H, H-2); 3.25-3.46 (m, 7H, H-3, H-4, H-5, $N^+C\underline{H}_2CH_2$ CH_2); 3.52-3.69 (m, 11H, H-6(1H), OCH_2CH_2O, $OCH_2C\underline{H}_2N^+$); 3.83-3.92 (m, 5H, H-6(1H), C=$CNCH_2C\underline{H}_2O$, $OC\underline{H}_2CH_2N^+$); 4.41 (d, $^3J_{HH}$ = 7.95 Hz, 1H, H-1); 4.58 (t, $^3J_{HH}$ = 6.40 Hz, 2H, C=$CNC\underline{H}_2CH_2O$);

ACCEPTED MANUSCRIPT

4.76-4.97 (m, 2H, OCH_2N); 8.15 (s, 1H, s, 1H, HC=C). ^{13}C-NMR (δ CD_3OD, 100 MHz) ppm: 14.44; 23.25; 23.69; 27.34; 30.19; 30.44; 30.51; 30.62; 30.72; 30.73; 33.03; 51.39; 62.20; 62.71; 63.00; 63.72; 65.59; 70.31; 71.35; 71.40; 71.49; 71.59; 75.02; 77.96; 78.01; 103.59; 126.04; 145.49. HRMS: calculated for $C_{42}H_{83}N_4O_9$ $[M\text{-}Br^-]^+$: 787.6160; found: 787.6185. Elemental analysis for C, H, N was within the theoretic value.

*Determination of critical micellar concentration (cmc) of amphiphile **3***

The *cmc* of amphiphile **3** was measured at 25 °C according to a reported procedure that exploits the variation in the intensity of the vibronic fine structure of pyrene monomer fluorescence upon association with micellar/vesicular aggregates.[24] Aqueous solutions (3 mL) of amphiphile **3** at concentrations between 10 μM and 80 μM were added to a defined amount of pyrene in order to obtain ~ 0.5 μM final concentration of pyrene (prepared from 7 μL of a 160 μM stock solution of pyrene in absolute ethanol dried by a nitrogen flux). The solutions were kept above 37 °C, under stirring, for 12 h. Emission spectra of the solutions were acquired in the range 350–450 nm (λ_{exc} = 335 nm). A sharp increase of the ratio of the third and first vibronic bands of pyrene I_3/I_1 (I_3 at 380 nm, I_1 at 370 nm) occurs at *cmc*.

*Binding of amphiphile **3** with FITC-Con A*

The capability of amphiphile **3** to interact with FITC-Con A was investigated at 25 °C by steady-state fluorescence experiments (at 525 nm; λ_{exc} = 488 nm) according to a reported procedure.[25] The variation of fluorescence intensity I 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 upon the addition of small volumes of a 14.2 mM aqueous solution of amphiphile **3** in PBS was monitored. In a blank experiment the variation of 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) upon the addition of small volumes of the aqueous solution of

ACCEPTED MANUSCRIPT

amphiphile **3** in PBS (to obtain concentrations of **3** analogous to those of the previous experiment) was monitored. The fluorescence spectra were recorded after equilibrium state was reached. All fluorescence intensities were normalized according to the dilution factor. The following equation was thus applied to evaluate the percentage of fluorescence increase due to displacement of glycogen ΔΔI(%):

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

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 **3**.

Liposomes preparation

Lipid films were prepared by evaporation of solutions containing the proper amount of DMPC (dissolved in $CHCl_3$) and **3** (dissolved in MeOH) to obtain the desired molar percentage mixture. For Tm determination of DMPC/**3** liposomes, Laurdan (dissolved in $CHCl_3$) was included in the lipid films. For liposome uptake by human breast cancer cells, NBD-PE (dissolved in $CHCl_3$) was added to the components of the lipid films. The lipid films were kept overnight under reduced pressure (0.4 mbar), then PBS was added to obtain a lipid dispersion of the desired concentration; liposome solutions were vortex-mixed and multilamellar vesicles (MLV) were obtained. MLV were freeze-thawed six times from liquid nitrogen to 50 °C and then extruded (10 times) through a 100 nm polycarbonate membrane.

Determination of aggregate size by DLS

ACCEPTED MANUSCRIPT

DLS measurements were carried out on aqueous solutions of amphiphile **3** (80 mM) and suspensions of DMPC/**3** liposomes at molar ratios 95:5, 80:20, 70:30 (1.25 mM total lipids in PBS) soon after preparation and after 48 hours.

Tm determination by fluorescence experiments

Tm of DMPC/**3** (95:5, 80:20, 70:30) liposome formulations was investigated by fluorescence experiment applying an indirect method described in the literature that exploits Laurdan as fluorescent probe (DMPC/**3**/Laurdan, 95:5:0.1).[26] Emission spectra (λ_{exc} = 360 nm) of liposome suspension (1 mM total concentration) in PBS were acquired varying the temperature in the range 15-35 °C. The temperature of the sample was measured by a thermocouple. Equation 2 provides the generalized polarization parameter (GP):

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

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 Tm value corresponds to the inflection point of the sigmoidal curve obtained by plotting the GP versus the temperature of the sample. Error in determination is of ± 0.3 °C.

*Determination of thermotropic properties of DMPC/**3** liposomes*

DSC measurements were carried out on 30 μL of MLV (1 mg/10 μL, 148 mM total lipids). Two heating scans were recorded at the rate of 5 °C/min and two subsequent heating scans were recorded at the rate 1 °C/min. Under the experimental conditions, reproducible thermal recordings were obtained. Uncertainty on temperatures was determined to be 0.1 °C.

Determination of surface potential of glucosylated liposomes

ACCEPTED MANUSCRIPT

Surface potential (ψ°) of DMPC/**3** liposomes was determined by an indirect method using HC, a pH-sensitive fluorescent probe.[27] Excitation spectra of 5 mM liposome suspension between 300 and 400 nm (emission wavelength = 450 nm) were recorded. Fluorescence measurements of DMPC/**3**/HC (95:5:0.3) in PBS were performed by scanning the excitation wavelength upon variation of the pH of solution in the 2-12 range (obtained by addition of aqueous sodium hydroxide or hydrochloric acid). The extent of dissociation of HC included in the lipid bilayer was monitored by the ratio of the excitation fluorescence intensities at 380 and 330 nm (pH-independent isosbestic point). pKa of HC associated with cationic lipid bilayer (pKa charged) corresponds to the inflection point of the plot of I380/I330 ratio *versus* pH. The ψ° was obtained by a conversion and rearrangement of the Boltzmann equation:

$$\psi^\circ = (\mathrm{p}K_a^{charged} - \mathrm{p}K_a^{neutral}) k_B T \ln 10/e$$

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

Agglutination of glucosylated liposomes by Con A monitored by OD measurements

The variation of specific turbidity of a 3 mL sample of 0.83 mM DMPC/**3** (95:5, 80:20 and 70:30 molar ratios) liposomes in PBS upon the addition of Con A (0.33 mg/mL) was recorded over 1000 minutes. Scans were carried out on liposome PBS suspension in a 1 cm quartz cell upon addition of Con A, at 525 nm, immediately after mixing and every minute.

Cell culture

Human breast cancer cell lines MCF7, MDA-MB-231 and SKBR3 were grown as monolayer in DMEM supplemented with 10% FBS, 1% penicillin (50 IU/mL) and streptomycin (50 IU/mL) in a humidified atmosphere of 5% CO_2 in a water-jacketed incubator at 37 °C.

Flow Cytometry

ACCEPTED MANUSCRIPT

The uptake of liposomes, fluorescently labeled with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) tagged lipid NBD-PE, was evaluated by flow cytometry after 30 min, 1h, 4h and 18 h of treatment with 20 μM suspensions of the following liposome formulations: (i) DMPC/**1** 95:5, (ii) DMPC/**2** 95:5, (iii) DMPC/**3** 95:5 and (iv) DMPC/**3** 7:3. After treatment, cells were washed with ice-cold PBS, detached with EDTA and trypsin, resuspended in ice cold PBS and immediately analyzed. Fluorescence signals were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with a 15 mW, 488 nm, and air-cooled argon ion laser. The NBD-PE fluorescent emission was collected through a 530 nm band pass filter. At least 10,000 events were analyzed. Liposome uptake was evaluated as fluorescence intensity and expressed in arbitrary units (AU), calculated as the ratio between the mean fluorescence channel (MFC) of treated cells and the MFC of untreated cells. The analysis was performed by CellQuest software (Becton Dickinson).

Laser Scanning Confocal Microscopy

Cells, grown on 12 mm glass coverslips, were inoculated with liposome formulations. Intracellular distribution of fluorescently labeled liposomes was analyzed by Laser Scanning Confocal Microscopy (LSCM) after 18 h of treatment. After treatment cells were fixed in 3.7% paraformaldehyde in PBS, for 10 minutes at room temperature. Observations were performed by a Leica TCS SP2 laser scanning confocal microscopy (Leica, Microsystems, Mannheim, Germany) equipped with an Ar/Kr laser.

ACCEPTED MANUSCRIPT

RESULTS

*Amphiphile **3** properties*

Krafft point and Krafft temperature of **3** were evaluated as <4 °C because a 20 mM aqueous solution of **3** (above *cmc*) is completely transparent and stable at 4 °C. The *cmc* of amphiphile **3** in deionized water at 25 °C, determined by a fluorimetric method that exploits pyrene as fluorescent probe, was found to be $2.5 \cdot 10^{-5}$ M.

*Interaction of amphiphile **3** with FITC-Con A*

It is known that the fluorescence of FITC-Con A is quenched upon complexation with multibranched polysaccharides of D-glucose, such as glycogen, and can be restored by addition of monomeric D-glucose.[25] The fluorescence of FITC-Con A was restored upon the addition of **3** to a PBS solution containing the FITC-Con A/glycogen conjugate because the glycosylated lipid displaced glycogen from the binding sites of FITC-Con A (Figure SI 1).

*Size and size distribution of **3** and of DMPC/**3** liposomes*

DLS measurements on solution of pure **3** showed that this amphiphile forms aggregates of ~6 nm diameter. The DLS analysis of DMPC/**3** liposomes at 95:5, 80:20 and 70:30 molar ratios were performed soon after extrusion and after 48 hours; results indicated the presence of stable monomodal distributions of liposomes featuring a hydrodynamic diameter of ~120, 105 and 165 nm, respectively (PDI lower than 0.2).

*Tm of DMPC/**3** liposomes and relative transition enthalpies*

The values of Tm (obtained by a fluorimetric method and by DSC) and relative transition enthalpies (obtained by DSC) for DMPC/**3** formulations are reported in Table 1.

ACCEPTED MANUSCRIPT

Table 1. Tm values and relative transition enthalpies for DMPC and DMPC/**3** liposomes at molar ratios 95:5, 80: 20 and 70:30. Uncertainty in determination is ±0.5°C, ±0.1°C and ±0.5 kJ/mol for a, b, and c entries, respectively.

Formulation	Tm / °C [Laurdan][a]	Tm / °C [DSC][b]	ΔH^c / kJ mol^{-1}
DMPC		24.1	28.9
DMPC/**3** (95:5)	24.6	23.8	27.8
DMPC/**3** (80:20)	21.8	22.5	8.5
DMPC/**3** (70:30)	22.2	-	-

*Thermotropic properties of DMPC/**3** liposomes*

Thermograms and Tm and ΔH values obtained by DSC measurements are reported in Figure 1 and Table 1, respectively. The value of Tm of DMPC/**3** liposomes at 95:5 molar ratio is very close to the Tm of liposomes of mere DMPC whereas the corresponding peak on the thermogram is considerably broader. When **3** is included in DMPC liposomes at 20% molar percentage the peak corresponding to the main transition is broader with respect to DMPC liposomes and both Tm and ΔH values are shifted to lower values whereas when **3** is included at 30% molar percentage the transition is not observable.

ACCEPTED MANUSCRIPT

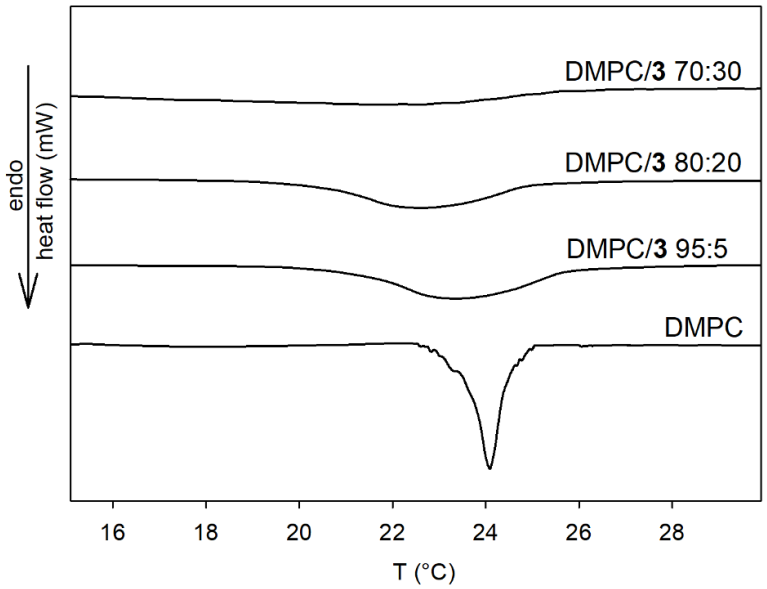


Figure 1. Thermograms relative to various mole ratios DMPC/**3** MLVs organized according to increasing percentages of GA **3**, the first thermogram from the bottom being relative to vesicles formulated only with DMPC. Scan rate is 1 °C/min.

*Surface potential of DMPC/**3** liposomes*

DMPC/**3** liposomes at molar ratios 95:5, 80: 20 and 70:30 feature, as expected, a positive surface potential whose value increases at increasing percentage of **3** in the formulation, 26 mV, 56 mV, and 95 mV, respectively.

*Agglutination of DMPC/**3** liposomes by Con A monitored by OD measurements*

The addition of Con A to DMPC/**3** formulations 95:5 and 80:20 yielded an increase of OD of the suspensions (Figure 2), whereas in the case of DMPC/**3** 70:30 agglutination was not observed. Figure 2 shows that agglutination of DMPC/**3** at 80:20 lipid ratio occurs faster and to a higher extent with respect to 95:5. In the case of DMPC/**3** 95:5 a decrease of turbidity was observed after ~ 600 minutes, due to precipitation of agglutinated particles; note that in the

ACCEPTED MANUSCRIPT

absence of Con A or in the case of not functionalized DMPC vesicles, no significant OD changes were detected within 1000 minutes.

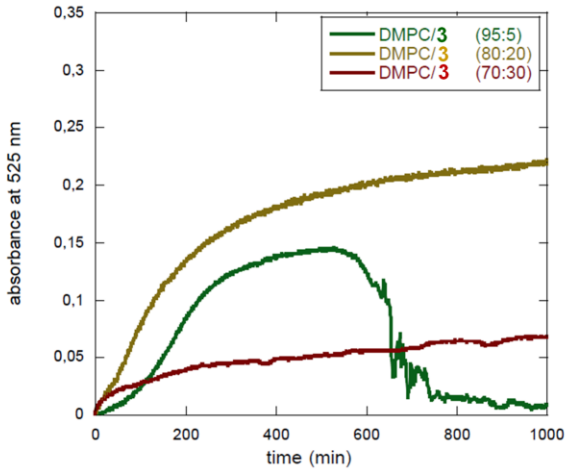


Figure 2. Effect of the addition of Con A on the optical density, measured as absorbance at 525 nm, of a suspension of DMPC/**3** liposomes in PBS at lipid ratios of 95:5, 80:20 and 70:30. Con A concentration 0.33 mg/mL; total lipids concentration 0.83 mM.

Cell ~~uptake~~ association of glycosilated liposomes

Liposome-cell association was evaluated by flow cytometry at 30 min, 1h, 4h and 18 hr. No significant differences were observed between liposome formulations after 30 min and 1 h (data not shown). Flow cytometry analysis, reported in Figure 3, shows the association (expressed as arbitrary units, A.U.) of liposomes with breast cancer cells. After 4 h of treatment, MFC values revealed in all cell lines incubated with DMPC/**1** 95:5, DMPC/**2** 95:5 and DMPC/**3** 95:5 liposomes did not significantly differ from those of cells treated with DMPC liposomes. On the other hand, a significant increase of MFC values was detected in MDA and SKBR3 cell lines treated with DMPC/**3** liposomes at 70:30 molar ratio.

The analysis performed after 18 h of treatment showed an increase of A.U. values in cells treated with DMPC/**1** 95:5 and DMPC/**2** 95:5 formulations when compared to cells treated with

ACCEPTED MANUSCRIPT

DMPC liposomes. No significant differences were observed between A.U. values of samples treated with DMPC/**3** 95:5 formulation and those of cells treated with DMPC liposomes. On the other hand, A.U. values of cells significantly increased after the incubation with DMPC/**3** 70:30 liposomes in all three breast cancer cell lines.

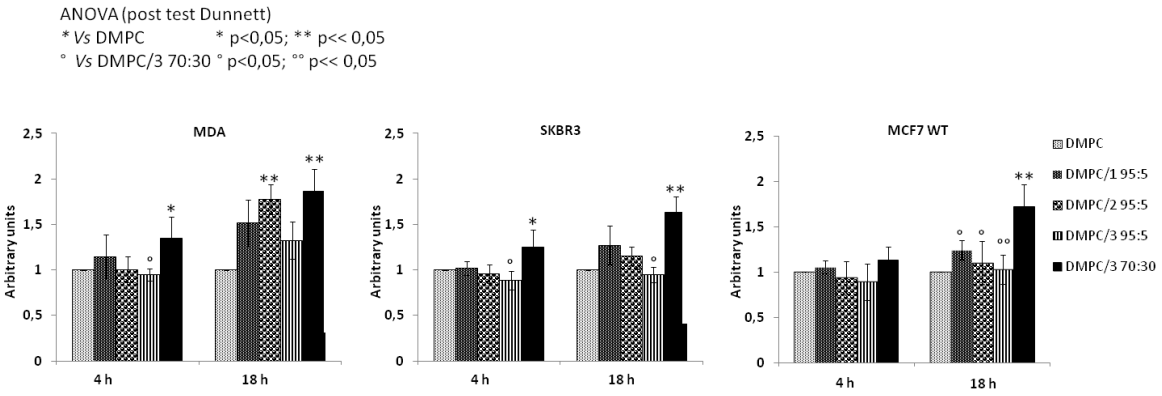


Figure 3. Association of different glucosylated liposomes with three different human breast cancer cell lines.

Intracellular distribution of glycosilated liposomes

The observations performed by LSCM after 18h of treatment showed different cell distributions of the evaluated liposome formulations (green signal, Figure 4). Generally, in agreement with flow cytometry data, MDA-MB-231 cells resulted the most efficient in the internalization of liposomes. In all cell lines DMPC, DMPC/**1** 95:5 and DMPC/**2** 95:5 liposomes appeared to be distributed as fluorescent spots, very likely in cytoplasmic vesicles localized in the perinuclear region. On the other hand, DMPC/**3** 70:30 liposomes appeared strongly clustered in the proximity of the plasma membrane.

ACCEPTED MANUSCRIPT

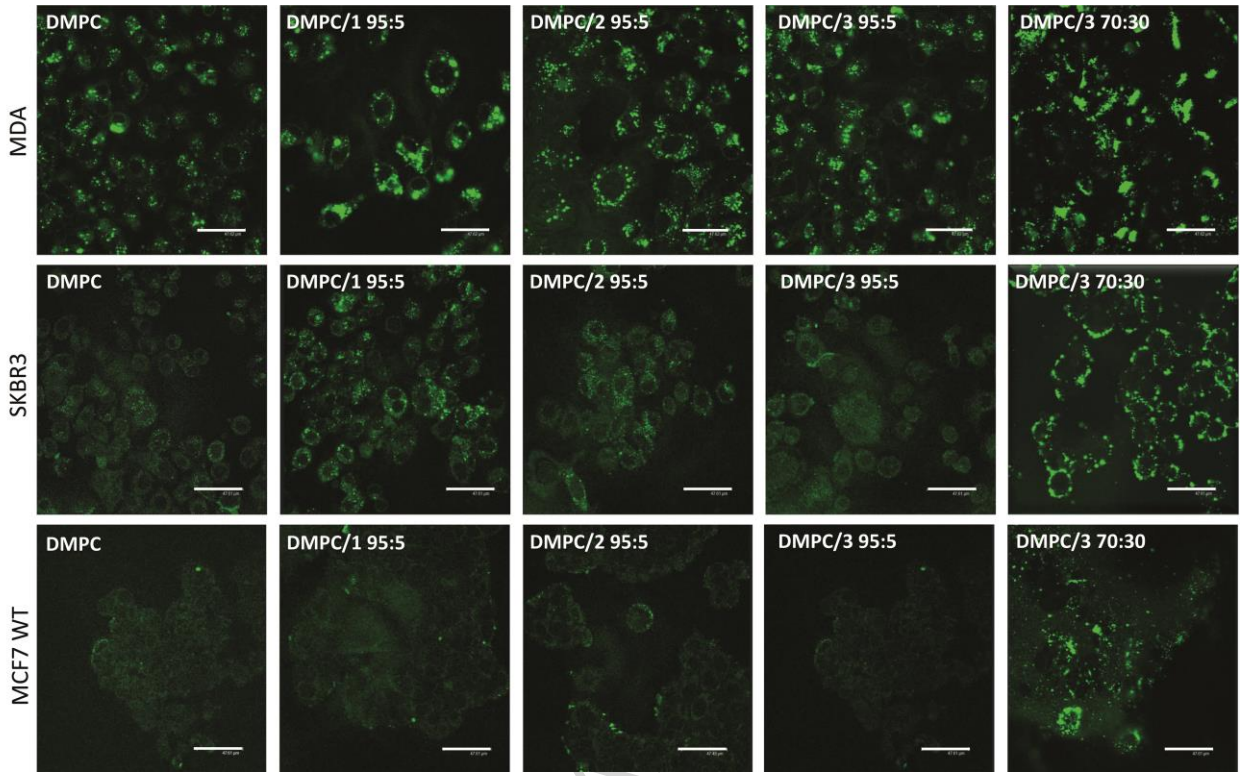


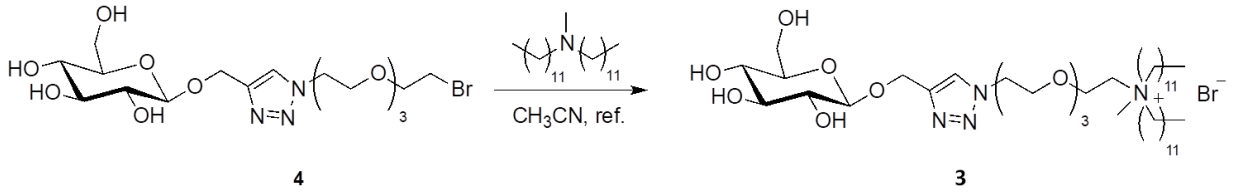
Figure 4. Images obtained by LSCM analysis after 18h of treatment on three cells lines with different DMPC/GA liposomes at 95:5 molar ratio and DMPC/**3** at 70:30 molar ratio.

DISCUSSION

*Physicochemical characterization of amphiphile **3***

Amphiphile **3** was obtained by alkylation of *N*,*N*-didodecylmethylamine with compound **4** in anhydrous CH_3CN (Scheme 1). The preparation of the hydrophilic alkylating residue **4** was previously described,[22] whereas *N*,*N*-didodecymethylamine was obtained by alkylation of *N*-dodecylmethylamine with 1-bromododecane in absolute EtOH.

ACCEPTED MANUSCRIPT



Scheme 1. Synthesis of amphiphile **3**.

The hydrophobic portion of **3** is considerably larger with respect to the two analogues **1** and **2** investigated previously. This structural difference can influence its aggregation features;[28] actually the aim of this variation in the molecular structure of the glycosylated lipid component was reducing the detergent capability of the amphiphile in order to increase its amount in the formulation with phospholipids glycosylated component in mixed liposomes. In fact in the case of **1** and **2** their amount in formulation with DMPC can not exceed 5% due to their detergent properties.[22,23] Further, the different hydrophobic portion might affects other properties of lipid membrane, such as lipid packing, fluidity, permeability and, as a consequence, their interaction with cells.[29] The DLS analysis of aggregates of pure **3** suggested that this amphiphile forms micelles. This behavior was unexpected, because in general twin type amphiphiles form vesicles,[28] however it can be explained considering that the polar headgroup is quite large and the balance between the hydrophobic and hydrophilic region of the amphiphile, hence the packing parameter, addresses aggregation towards micelles rather than vesicles. In the case of DMPC/**3** liposomes the large headgroup of glycolipid is counterbalanced by the smaller headgroup of DMPC and the formation of liposomes is not hampered. All the investigated mixed formulations showed a diameter in good agreement with the dimension imposed by the extrusion protocols. Differently from what observed in the case of DMPC/**1** and DMPC/**2** liposomes that were monomodal and stable only at 95:5 molar ratio,[22,23] **3** can be included up to 30% in DMPC

ACCEPTED MANUSCRIPT

liposomes due to the low detergent effect attributed by the presence of twin tails. These results indicate that, though **3** is a micelle-forming amphiphile, its twin tails confer it a less detergent effect with respect to its structural single tail analogues **1** and **2**.

The results obtained by DSC measurements clearly indicate that the presence of amphiphile **3** in DMPC formulations, even at very low percentages, significantly loosens lipid packing. Yet at 5% of **3** in the formulation Tm and ΔH are shifted to lower values and the cooperativity of the transitions is sensibly reduced (as reflected by the width of the peak) in analogy with DMPC/**1** and DMPC/**2** liposomes at the same molar ratio.[23] The disturbing effect of **3** on lipid packing increases as a function of its amount in the formulation. In fact, in the case of 80:20 DMPC/**3** the transition is still observable, but the peak is significantly broadened and both Tm and ΔH values decrease. The fact that the peak relative to the main transition is practically absent in the case of 70:30 DMPC/**3** liposomes indicates that lipid packing is slackened and lipid bilayer is probably very leaky. However, despite its destabilizing action, these results confirm that the detergent effect of **3** is lower with respect to that of **1** and **2**. This is certainly due to the higher number of van der Waals contacts of its large hydrophobic portion.

The capability of **3** (as monomer or included in mixed liposome) to interact with lectins was investigated using as a model Con A, a homotetrameric protein that bears four binding sites for glucose. Fluorescence experiments indicated the capability of **3** to bind with Con A due to its glucose moiety because the addition of **3** to a PBS solution of FITC-Con A/glycogen conjugate displaced glycogen thus restoring the fluorescence of FITC-ConA (Figure SI 1), similarly to what reported in the case of **1** or **2**.[23] However, in the experiments concerning **1** and **2** the complete restoration of fluorescence signal was observed at lower concentration of the glucosylated component (≈ 1mM *vs* 5 mM). This suggests that the bulkiness of the hydrophobic

ACCEPTED MANUSCRIPT

portion of **3** might hamper the interaction of the sugar moiety with the binding sites of the lectin. In fact in this experiment the glucosylated amphiphile should displace glycogen, a bulky multibranched polysaccharide, conjugate with FITC-Con A. It is thus reasonable that steric hindrance of **3** might slow down the completion of the process.

In the case of DMPC/**3** liposomes the binding of glucose moiety with Con A was evaluated by following by OD the eventual agglutination upon the addition of the protein to the liposome suspension: the increase of the OD of DMPC/**3** liposome suspensions (at 95:5, 80:20 and 70:30 molar ratios) indicates the increase of particle size due to agglutination. The decrease of OD observed in the case of 95:5 DMPC/**3** liposomes at ~ 600 minutes clearly indicates particle precipitation. Results clearly indicate that the extent of agglutination decrease by increasing the amount of GA. This apparent incongruity can be explained considering that the presence of a high amount of **3** in formulation disturbs lipid packing, as indicated by DSC results, and that the defects formed on the surface of vesicles might accommodate the sugar moiety. Analogous observation was reported in the case of a pH sensitive glucosylated amphiphile.[30] The role of lipid compaction on the exposure of glucose moiety in the bulk is supported by the comparison of agglutination and DSC experiment results obtained in the case of 95:5 DMPC/**3** liposomes with the results of analogous agglutination and DSC experiments reported previously concerning 95:5 DMPC/**1** and DMPC/**2** liposomes. In fact it was observed that 95:5 DMPC/**3** liposomes feature the slowest and lowest extent of OD increase (Figure 5A), in correspondence with the lowest compaction of lipid bilayer as shown by thermograms (Figure 5B).[23] Further, a previous investigation on the influence of the state of phase of lipid membrane on the exposure of glucose residues on the surface of liposomes also showed the crucial role of lipid packing on the control of the interaction with lectins,[31] due to the housing of sugar moieties in the voids formed by loose

ACCEPTED MANUSCRIPT

lipid packing. Therefore, proper surface functionalization (ligand connected to a proper hydrophilic spacer) might not guarantee targeting, without a control on the state of phase and on the organization of lipid membrane.

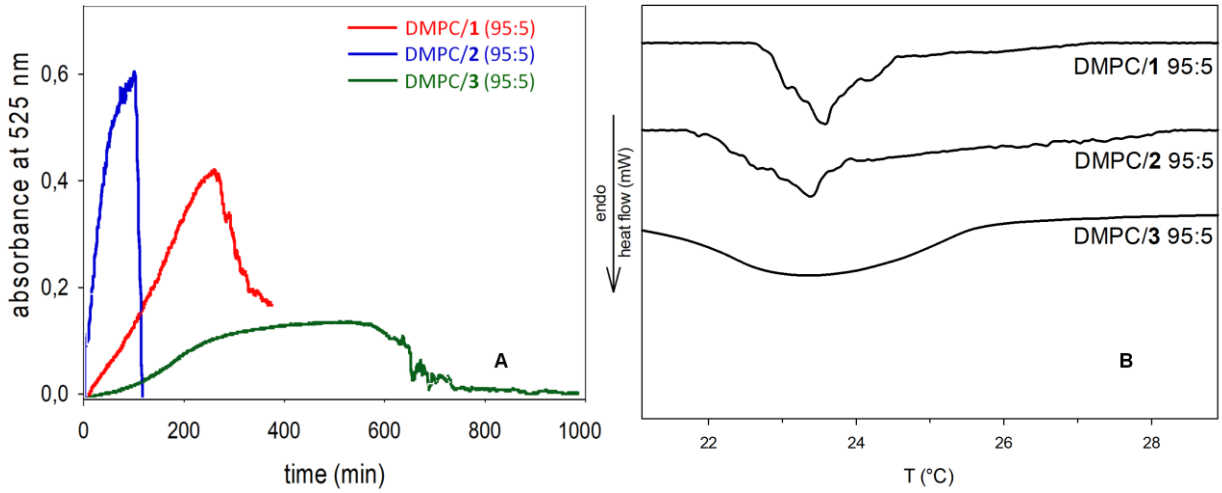


Figure 5. A) Effect of the addition of Con A on the optical density, measured as absorbance @ 525 nm, of suspensions of DMPC/**1**, DMPC/**2** and DMPC/**3** liposomes in PBS at lipid ratio 95:5. ConA concentration 0.33 mg/mL; total lipids concentration 0.83 mM. B) Thermograms relative to DMPC/**1** (top), DMPC/**2** (middle), DMPC/**3** (bottom) MLV. Scan rate is 1◦C/min. Data for DMPC/**1** and DMPC/**2** liposomes are taken from ref. 23.

Biological investigation

The targeting efficacy of glucosylated liposomes DMPC/**1**, DMPC/**2** and DMPC/**3** was evaluated on three human breast cancer cell lines, which express glucose receptor, namely GLUT1.[32] GLUT1 is present at variable levels in many tissues and is believed to be responsible for basal glucose uptake.[33,34] Elevated GLUT1 expression has been described in many cancers, including hepatic, pancreatic, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian, and breast carcinoma.[35,36] GLUT1 expression in breast cancer has been extensively studied also in patients and has been correlated with poor prognosis.[37] Thus, breast cancer cells represent an

ACCEPTED MANUSCRIPT

optimal model to investigate the targeting features of glucosylated liposomes. The expression of GLUT1 in the selected cell lines was checked before the investigation (data not shown).

Flow cytometry experiments (cell-liposome association) and LSCM observations (liposome internalization) clearly indicated that (i) the three cell lines feature different cell-association and cell-internalization, and (ii) DMPC/**3** liposomes show a different behaviour with respect to DMPC/**1** and DMPC/**2** liposomes.

The differences in the cellular association among the three breast cancer cell lines are very likely due to the different composition of their plasma membrane (*i.e*. lipid composition, proteins such as glucose receptors, etc).[38] The different behaviour of the formulations in the interaction with each cell line is mainly controlled by the hydrophobic portion of the glucosylated component and, as a consequence, by lipid organization. In fact, in the case of 95:5 formulations, those containing **1** and **2** interact more efficiently with cells with respect to liposomes containing **3**. Though liposome-cell association significantly increases in the case of 70:30 DMPC/**3** liposomes, it is worth of note that the intracellular distribution of DMPC/**3** liposomes is in both cases (95:5 and 70:30) dramatically different from that of DMPC/**1** and DMPC/**2** ones. In fact DMPC/**3** liposomes result mainly localized in the proximity of cell membrane, whereas DMPC/**1** and DMPC/**2** ones appear distributed in intracytoplasmic vesicles. The different uptake of DMPC/**3** liposomes could be ascribed to their reduced lipid packing with respect to DMPC/**1** and DMPC/**2** liposomes (as suggested by DSC results) that probably involves a different exposure of the glucose moiety and/or its inclusion in the voids present in lipid bilayer. Another possible reason of the different behavior of liposomes containing **3** with respect to those containing **1** or **2** could be the different fluidity/elasticity of lipid bilayer, parameter that can affect cell internalization of liposomes.

ACCEPTED MANUSCRIPT

CONCLUSIONS

The correlation of some physico-chemical properties of different mixed DMPC/GA liposomes with their ability to influence the uptake and the intracellular distribution in three breast cancer cell lines pointed out that the functionalization of liposomes with glycolipids can improve the interaction with cells. The modality and the extent of such interaction strongly depend on the molecular structure and/or the amount of the glucosylated component. The hydrophobic region of the lipid bilayer, in particular lipid packing, seems to play a pivotal role in the interaction of glycosylated liposomes with sugar receptors on target cells. As a whole, besides confirming that the decoration of liposomes surface with sugar residues has very good potential in active targeting, this investigation put in evidence how a proper design of the glycosylated component, even in the hydrophobic portion, is crucial to ensure the highest delivery efficiency.

AUTHOR INFORMATION

Corresponding Author

*giovanna.mancini@uniroma1.it

REFERENCES

(1) Lis, H.; Sharon, N. Lectins: Carbohydrate-specific proteins that mediate cellular recognition. *Chem. Rev*., **1998**, *98(2)*, 637-674.

(2) Luciani, A.; Olivier, J. C.; Clement, O.; Siauve, N.; Brillet, P. Y.; Bessoud, B.; Gazeau, F.; Uchegbu, I. F.; Kahn, E.; Frij, G.; Cuenod, C. A. Glucose-receptor MR imaging of tumors: study in mice with PEGylated paramagnetic niosomes. *Radiology* **2004**, *231(1)*, 135-142.

ACCEPTED MANUSCRIPT

(3) Goodman, C. K.; Wolfenden, M. L.; Nangia-Makker, P.; Michel, A. K.; Raz, A.; Cloninger, M. J. Multivalent scaffolds induce galectin-3 aggregation into nanoparticles, *Beilstein J. Org. Chem*. **2014**, *10*, 1570-1577.

(4) Ulbrich, W.; Lamprecht, A. Targeted drug-delivery approaches by nanoparticulate carriers in the therapy of inflammatory diseases. *J. R. Soc. Interface* **2010**,*7*, S55-S66.

(5) Bies, C.; Lehr, C.-M.; Woodley, J. F. Lectin-mediated drug targeting: history and applications. *Adv. Drug Deliv. Rev.* **2004**, *56*, 425-435.

(6) Garg, M.; Dutta, T.; Jain, N. K. Reduced hepatic toxicity, enhanced cellular uptake and altered pharmacokinetics of stavudine loaded galactosylated liposomes. *Eur. J. Pharm. Biopharm*. 2007, *67*, 76-85.

(7) Sliedregt, L. A.; Rensen, P. C.; Rump, E. T.; van Santbrink, P. J.; Bijsterbosch, M. K.; Valentijn, A. R.; van der Marel, G. A.; van Boom, J. H.; van Berkel, T. J.; Biessen, E. A. Design and synthesis of novel amphiphilic dendritic galactosides for selective targeting of liposomes to the hepatic asialoglycoprotein receptor. *J. Med. Chem*. **1999**, *42*, 609-618.

(8) Oh, H. R.; Jo, H.-Y.; Park, J. S.; Kim, D.-E.; Cho, J-Y.; Kim, P.-H.; Kim, K.-S. Galactosylated Liposomes for Targeted Co-Delivery of Doxorubicin/Vimentin siRNA to Hepatocellular Carcinoma. *Nanomaterials* **2016**, *6(8)*, 141-157.

(9) Zhao, C.; Feng, Q.; Dou, Z.; Yuan, W.; Sui, C.; Zhang, X.; Xia, G.; Sun, H.; Ma, J. Local targeted therapy of liver metastasis from colon cancer by galactosylated liposome encapsulated with doxorubicin. *PLoS One* **2013**, *8(9)*, e73860

ACCEPTED MANUSCRIPT

(10) Engel, A.; Chatterjee, S. K.; Ali-arifi, A.; Riemann, D.; Langner, J. Nuhn, P. Influence of spacer length on interaction of mannosylated liposomes with human phagocytic cells. Pharm. Res. **2003**, *20(1)*, 51-57.

(11) Kawakami, S.; Sato, A.; Nishikawa, M.; Yamashita, F.; Hashida, M. Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes, *Gene Ther*. **2000**, *7*, 292-299.

(12) Opanasopit, P.; Sakai, M.; Nishikawa, M.; Kawakami, S.; Yamashita, F.; Hashida, M. Inhibition of liver metastasis by targeting of immunomodulators using mannosylated liposome carriers. *J. Control. Release* **2002**, *80*, (2002) 283-294.

(13) Y. Hattori, S. Suzuki, S. Kawakami, F. Yamashita, M. Hashida, The role of dioleoylphosphatidylethanolamine (DOPE) in targeted gene delivery with mannosylated cationic liposomes via intravenous route, *J. Control. Release* **2005**, *108*, 484-495.

(14) Higuchi, Y.; Kawakami, S.; Oka, M.; Yabe, Y.; Yamashita, F.; Hashida, M. Intravenous administration of mannosylated cationic liposome/NFkappaB decoy complexes effectively prevent LPS-induced cytokine production in a murine liver failure model, FEBS Lett. **2006**, *580,* 3706-3714.

(15) Kawakami, S.; Wong, J.; Sato, A.; Hattori, Y.; Yamashita, F.; Hashida, M. Biodistribution characteristics of mannosylated, fucosylated, and galactosylated liposomes in mice, *Biochim. Biophys. Acta* **2000**, *1524*, 258-265.

(16) Ohannesian, D. W.; Lotan, R. In *Glycosciences: Status and Perspectives*; Gabius, H.-J., Gabius, S., Eds.; Chapman & Hall: Weinheim, 1997; p 459.

ACCEPTED MANUSCRIPT

(17) Akahani, S.; Inohara, H.; Mangia-Makker, P.; Raz, A. Galectin-3 in tumor metastasis. *Trends Glycosci. Glycotechnol.* **1997**, *9*, 69-75.

(18) Dwek, R. A.;Butters, T. D.; Platt, F. M.; Zitzmann, N. Targeting glycosylation as a therapeutic approach. *Nat. Rev. Drug Discov.* **2002**, *1*, 65-75.

(19) Engel, A.; Chatterjee, S. K.; Al-Arifi, A.; Nuhn, P. Influence of spacer length on the agglutination of glycolipid-incorporated liposomes by ConA as model membrane. *J Pharm Sci*. **2003**, *92(11)*, 2229-2235.

(20) Chabre, Y.M.; Roy, R. Design and creativity in synthesis of multivalent neoglycoconjugates. *Adv. Carbohydr. Chem. Biochem*. **2010**, *63*,165-393.

(21) Jayaraman, N.; Maiti, K.; Naresh, K. Multivalent glycoliposomes and micelles to study carbohydrate–protein and carbohydrate–carbohydrate interactions. *Chem. Soc. Rev*. **2013**, *42*, 4640-4656.

(22) Mauceri, A.; Borocci, S.; Galantini, L.; Giansanti, L.; Mancini, G.; Martino, A.; Salvati Manni, L.; Sperduto, C. Recognition of concanavalin A by cationic glucosylated liposomes. *Langmuir* **2014**, *30*, 11301-11306.

(23) Mauceri, A.; Fracassi, A.; D'Abramo, M.; Borocci, S.; Giansanti, L.; Piozzi, A.; Galantini, L.; Martino, A.; D'Aiuto, V.; Mancini, G.. Role of the hydrophilic spacer of glucosylated amphiphiles included in liposome formulations in the recognition of Concanavalin A. *Colloids Surf. B Biointerfaces* **2015**, *136*, 232–239.

(24) Goddard, E. D.; Turro, N. J.; Kuo, P.L.; Ananthapadmanabhan, K. P. Fluorescence probes for critical micelle concentration determination. *Langmuir* **1985**, *1*,352-355.

ACCEPTED MANUSCRIPT

(25) Sato, K.; Anzai, J. Fluorometric Determination of sugars using fluorescein-labeled concanavalin a-glycogen conjugates, *Anal. Bioanal. Chem*. **2006**, *384*, 1297-1301.

(26) Parasassi, T.; De Stasio, G.; D'Ubaldo A.; Gratton, E. Phase fluctuation in phospholipid membranes revealed by Laurdan fluorescence. *Biophys. J*. **1990**, *57*, 1179-1186.

(27) Zuidan, N. J.; Barenholz, Y. Electrostatic parameters of cationic liposomes commonly used for gene delivery as determined by 4-heptadecyl-7-hydroxycoumarin. *Biochim. Biophys. Acta, Biomembr*.**1997**, *1329*, 211−222.

(28) Nagarajan, R.. Molecular packing parameter and surfactant self-assembly: the neglected role of the surfactant tail *Langmuir* **2002**, *18,* 31-38**.**

(29) Bnyan, R.; Khan, I.; Ehtezazi, T.; Saleem, I.; Gordon, S.; O'Neill, F.; Roberts, M. Surfactant effects on lipid-based vesicles properties. J. Pharm. Sci. **2018** , *107(5)*, 1237-1246.

(30) Giansanti, L.; Mauceri, A.; Galantini, L.; Altieri, B.; Piozzi, A.; Mancini, G. Glucosylated pH-sensitive liposomes as potential drug delivery systems. Chem. Phys. Lip. **2016**, *200*, 113-119.

(31) Gradella Villalva, D.; Giansanti, L.; Mauceri, A.; Ceccacci, F.; Mancini, G. Influence of the state of phase of lipid bilayer on the exposure of glucose residues on the surface of liposomes. *Colloids Surf. B Biointerfaces* **2017**, *159*, 557-563.

(32) Laudański, P.; Swiatecka, J.; Kovalchuk, O.; Wołczyński, S. Expression of GLUT1 gene in breast cancer cell lines MCF-7 and MDA-MB-231. *Ginekol. Pol*. **2003**, *74(9), 782-785.*

ACCEPTED MANUSCRIPT

(33) Birnbaum, M.J.; Haspel, H.C.; Rosen, O.M. Cloning and characterization of a cDNA encoding the rat brain glucose-transporter protein. *Proc Natl Acad Sci. USA*. **1986**, *83(16), 5784-5788.*

(34) Fukumoto, H.; Seino, S.; Imura, H.; Seino, Y.; Eddy, R.L.; Fukushima, Y.; Byers, M.G.; Shows, T.B.; Bell, G.I. Sequence, tissue distribution, and chromosomal localization of mRNAencoding a human glucose transporter-like protein. *Proc Natl Acad Sci USA*. **1988**, 85(15), 5434-5438.

(35) Yamamoto, T.; Seino, Y.; Fukumoto, H.; Koh, G.; Yano, H.; Inagaki, N.; Yamada, Y.; Inoue, K.; Manabe, T.; Imura, H. Over-expression of facilitative glucose transporter genes in human cancer. *Biochem. Biophys. Res Commun*. **1990**, *170, 223-230*.

(36) Carvalho, K.C.; Cunha, I.W.; Rocha, R.M.; Ayala, F.R.; Cajaíba, M.M.; Begnami, M.D.; Vilela, R.S.; Paiva, G.R.; Andrade, R.G.; Soares, F.A. GLUT1 expression in malignant tumors and its use as an immunodiagnostic marker. *Clinics* **2011**, 66(6), 965-972.

(37) Yu, M.; Yongzhi, H.; Chen, S.; Luo, X.; Lin, Y.; Zhou, Y.; Jin, H.; Hou, B.; Deng, Y.; Tu, L.; Jian, Z. The prognostic value of GLUT1 in cancers: a systematic review and meta-analysis*Oncotarget*. **2017**, 8(26) 43356-43367.

(38) He, M.; Guo, S.; Li. Z. *In situ* characterizing membrane lipid phenotype of breast cancer cells using mass spectrometry profiling. *Sci. Rep.* **2015**, *5*, article number: *11298, 1-13*.

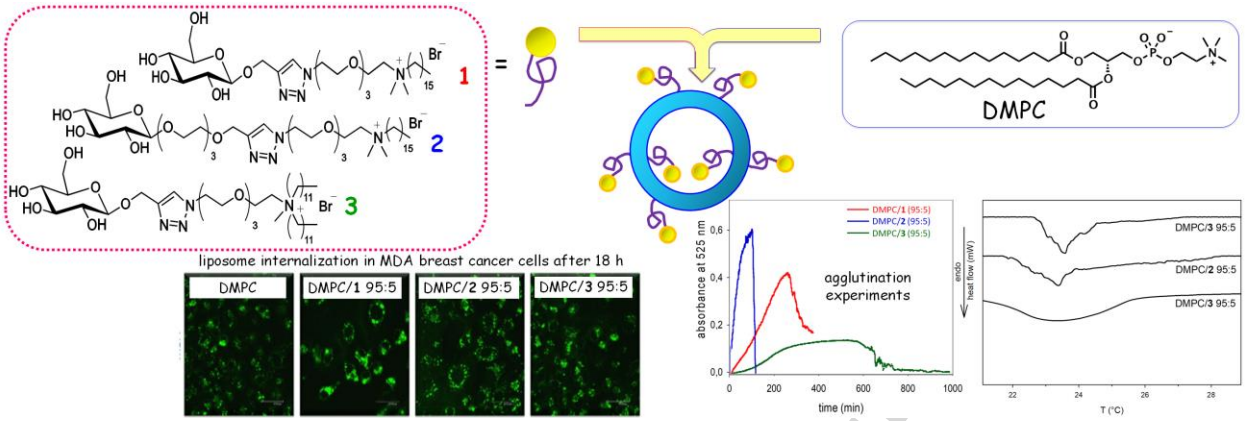
ACCEPTED MANUSCRIPT

Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT



Graphical abstract

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Highlights

- Liposomes containing conventional and twin glucosylated surfactants were studied.
- Also the length of the hydrophilic spacer of the glucosylated surfactants differ.
- The interaction of liposomes with three breast cancer cell lines was investigated.
- The hydrophobic portion of the bilayer is crucial for liposomes internalization.

ACCEPTED MANUSCRIPT