
Title: Glucosylated pH-sensitive liposomes as potential drug delivery systems

Author: Luisa Giansanti Alessandro Mauceri Luciano Galantini Barbara Altieri Antonella Piozzi Giovanna Mancini

PII: S0009-3084(16)30112-8
DOI: http://dx.doi.org/doi:10.1016/j.chemphyslip.2016.08.004
Reference: CPL 4490

To appear in: *Chemistry and Physics of Lipids*

Received date: 9-5-2016
Revised date: 26-7-2016
Accepted date: 31-8-2016

Please cite this article as: Giansanti, Luisa, Mauceri, Alessandro, Galantini, Luciano, Altieri, Barbara, Piozzi, Antonella, Mancini, Giovanna, Glucosylated pH-sensitive liposomes as potential drug delivery systems.Chemistry and Physics of Lipids http://dx.doi.org/10.1016/j.chemphyslip.2016.08.004

# Glucosylated pH-sensitive liposomes as potential drug delivery systems

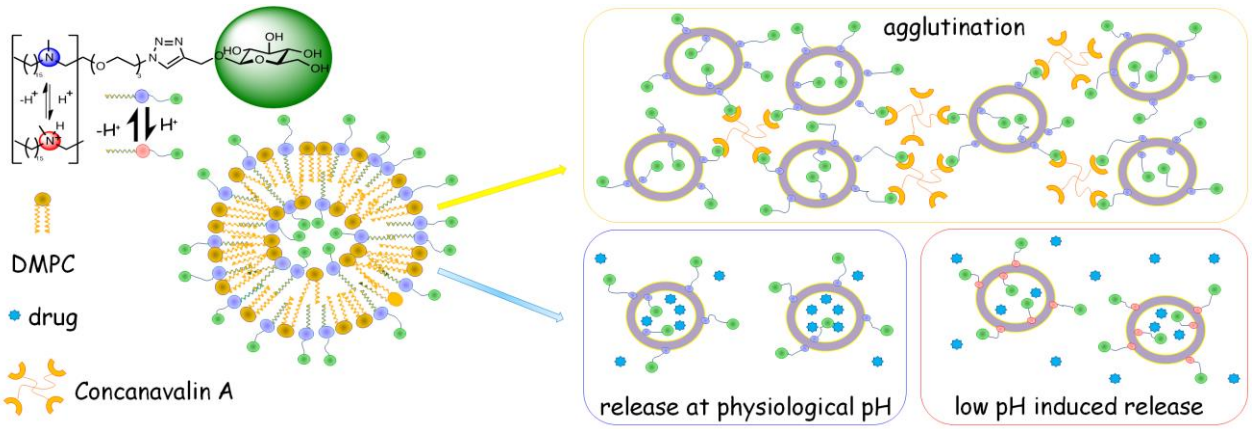
*Luisa Giansanti,*[a,b] *Alessandro Mauceri,* [b,c] *Luciano Galantini,*[c] *Barbara Altieri,*[a,b] *Antonella Piozzi,*[c] *Giovanna Mancini*[b,*]

[a]Dipartimento di Scienze Fisiche e Chimiche, Università degli Studi dell'Aquila, Via Vetoio, 67010 Coppito (AQ) (Italy)

[b] CNR, Istituto di Metodologie Chimiche, Area della Ricerca di Roma 1, Via Salaria km 29,300, 00015 Monterotondo (RM), Italy

[c] Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", P.le A. Moro 5, 00185 Roma (Italy)

Graphical abstract



Highlights

- A new glucosylated pH-sensitive amphiphile was prepared and characterized
- Glycosylated liposomes were investigated by DLS, DSC and fluorescence experiments
- pH-sensitive liposomes show different properties than their cationic analogues
- pH-sensitivity greatly influences lipid bilayer organization
- The interaction of glycosylated pH-sensitive liposomes with Concanavalin A

ABSTRACT

The inclusion of pH-sensitive components in liposome formulations can allow a more controlled and efficient release in response to low pH typical of some pathological tissues and/or subcellular compartments. On the other hand decorating the surface of liposomes with sugar moieties attributes to lipid vesicles specificity toward lectins, sugar-binding proteins overexpressed in many tumor tissues. A novel multifunctional pH-sensitive glucosylated amphiphile was synthesized and characterized as pure aggregate component and in mixtures with a natural phospholipid. The comparison of the properties of the new glucosylated amphiphile with respect to those of a previously described cationic structural analogue demonstrates that the pH-sensitivity can strongly affect drug release, lipid organization, as well as the exposure of the glucose residues on liposome surface and their ability to interact with Concanavalin A, a plant lectin used as model system.

**Keywords**: glucosylated amphiphile, pH-sensitivity, liposomes, agglutination, Concanavalin A, drug release

## 1. Introduction

Since ’70 liposomes have been widely investigated as drug delivery systems for their biocompatibility, efficiency and ability to entrap either hydrophilic or hydrophobic drugs and to improve their pharmacokinetic and pharmacodynamic profiles [1]. Liposomes mainly enter cells via endocytosys and if the drug does not escape the endosome there is a high risk of degradation with a

consequent reduction of its efficacy [2]. Different strategies have been proposed to avoid this phenomenon, and the use of pH-sensitive liposomes (*i.e.* liposomes containing a pH-sensitive component) is one of the most promising strategies. In fact, pH-sensitive liposomes are stable at physiological pH, whereas they can be destabilized and may show fusogenic properties under acidic conditions, typical of endosomal compartment and of certain target tissue such as tumour [3]. As a consequence of destabilization, the controlled release of the entrapped drug to the target tissue occurs, thus avoiding lysosomal sequestration and degradation. Moreover, it is possible to modify liposome surface to ascribe them certain features, such as increased circulation time in the bloodstream and specificity toward certain targets, even at subcellular level, thus improving their efficacy as drug delivery systems [4]. Over the last years many liposome formulations have been developed providing improved therapeutic outcomes and reduced side effects. Therefore liposomes with improved drug delivery potential for cancer therapy have been prepared, mainly based on the active targeting to tumor tissues (mainly by folate and transferring) and on the release of the chemotherapic agent triggered by pathological conditions typical of the microenvironment of tumor cells [5]. One of approaches relies on the use of glycosylated amphiphile that, when included in liposome formulations, increase their specificity to tumor tissue [6,7]. In fact, certain malignant tissues overexpress lectins that are sugar-binding proteins located at cell surface and involved in many recognition processes such as cell-cell adhesion, cell attachment to substratum and blood-borne metastasis [8].

The combination of pH sensitivity and specificity for target tissue can hugely increase the potential of liposomal formulations as delivery systems to cancer cells. Herein we report on the synthesis and the physicochemical characterization of a novel pH-sensitive glucosylated amphiphile (GA) **1** and on its employment as a component of liposome formulations. GA **1** is characterized by the presence of a PEG spacer that links a glucose moiety to a tertiary ammine group bearing a methyl and a hydrophobic alkyl chain. It is the pH-sensitive analogue of a previously reported cationic GA, **2**, that features a quaternary charged nitrogen as polar headgroup and that showed good ability to

interact with a plant lectin both as a monomer and as liposome component [9]. The aggregation properties of GA **1** were investigated as a pure component and when included in liposomes of 1,2-dimyristoyl-*sn*-glycero-phosphocholine, DMPC, (Chart 1) at different molar ratios. The effect of the inclusion of GA **1** on DMPC bilayer properties was studied by differential scanning calorimetry (DSC) measurements. The ability of the glucosylated liposomes to interact with lectins was evaluated by optical density (OD) measurement based agglutination assay monitoring their ability to bind Concanavalin A (ConA), a plant lectin usually used as model system in agglutination experiments. Finally the sensitivity of the formulations to pH conditions was investigated.

## 2. Materials and Methods

### *2.1. Materials*

DMPC was purchased from Avanti Polar Lipids (Alabaster, AL, USA). ConA, from *Canavalina ensiformis* (Jack bean), phosphate-buffered saline (PBS; Aldrich; 0.01 M phosphate buffer; 0.0027 M KCl; 0.137 M NaCl; pH 7.4) Sephadex G-50, 1-pyrenemethanol (pyr-m) and all reagents employed for the synthesis of **1** were purchased from Sigma-Aldrich. Compound **4** was prepared as previously reported [9]. All solvents and chemicals were used as purchased without further purification.

### *2.2. Instrumentation*

A Bruker300 Avance spectrometer (operating at 300 MHz for $^{1}$H and 75 MHz for $^{13}$C) was used to record NMR spectra. $^{1}$H resonances of deuterated solvents (with respect to TMS) were used as internal standards.

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 measurements.

A pHmeter Crison PH 25+ was used to measure the pH of the solutions.

A Malvern Nano-ZetaSizer spectrometer, equipped with a 5 mW HeNe laser ($\lambda_{ecc}$= 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 and analyzed by using the cumulant fit.

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

*2.3. Synthesis of GA **1***

GA **1** was synthesized following the procedure reported in Scheme 1.

*2.3.1 Preparation of N-methylhexadecanamide **2**.*

5 mL of aqueous $CH_3NH_2$ (40%) were added to 10 mL of $CH_2Cl_2$ and, after cooling at 0°C, the solution was magnetically stirred in a round bottom flask. 3 mL (10 mmol) of palmitoyl chloride were added dropwise and the mixture was left to react at room temperature for 24 h. The organic layer was extracted with aqueous NaOH 2 M, then with aqueous HCl 1 M and finally with a saturated aqueous solution of NaCl. The organic layer was dried over anhydrous $MgSO_4$ and, after filtration, the solvent was removed under reduced pressure to give 2.48 g of **2** (yield 92%) as a white solid. $^1$H-NMR (δ $CDCl_3$, 300 MHz) ppm: 5.79 (s, 1H, NH); 2.74-2.83 (os, 3H, $NCH_3$); 2.17 (t, $^3J_{HH}$ = 7.6 Hz, 2H, $COCH_2$); 1.54-1.72 (m, 2H, $COCH_2C\underline{H}_2$); 1.15-1.33 (m, 26H, $NCH_2CH_2[C\underline{H}_2]_{13}CH_3$); 0.87 (t, $^3J_{HH}$ = 6.5Hz, 3H, $CH_2C\underline{H}_3$). $^{13}$C-NMR (δ $CDCl_3$, 75 MHz) ppm: 175.16; 38.12; 33.27; 31.04; 31.00; 30.96; 30.84; 30.71; 27.61; 27.15; 24.04; 15.47.

*2.3.2 Preparation of N-methylhexadecanamine **3**.*

2.48 g (9.2 mmol) of *N*-methylhexadecanamide **2** were added in small portions to an ice-bath cooled suspension of 0.91 g (24.0 mmol) of $LiAlH_4$ in 100 mL of dry THF. The reaction was kept at reflux for 24 h. The mixture was then cooled at room temperature and 7 mL of 10% aqueous solution of NaOH, 5 mL of $H_2O$ and 80 mL of THF were added. The mixture was filtered and the solvent removed under reduced pressure. The oily residue was purified by chromatography on silica gel (eluent from $CHCl_3$ 100% to $CHCl_3$/MeOH = 80:20) to give 1.54 g of **3** (yield 65%) as a white

solid. $^{1}$H-NMR (δ $CDCl_3$, 300 MHz) ppm: 2.53 (t, $^{3}J_{HH}$ = 7.2 Hz, 2H, $NCH_2$); 2.40 (s, 3H, $NCH_3$); 2.12 (s, 1H, NH); 1.35-1.46 (m, 2H, $NCH_2C\underline{H}_2$); 1.09-1.31 (m, 26H, $NCH_2CH_2[C\underline{H}_2]_{13}CH_3$); 0.85 (t, $^{3}J_{HH}$ = 6.6 Hz, 3H, $CH_2C\underline{H}_3$). $^{13}$C-NMR (δ $CDCl_3$, 75 MHz) ppm: 53.32; 37.56; 33.27; 31.04; 30.97; 30.91; 30.71; 28.66; 24.03; 15.45.

*2.3.2 Preparation of GA **1***

0.34 g (1.3 mmol) of *N*-methylhexadecanamine **3** and 0.45 g (0.9 mmol) of **4** were suspended in 8 mL of dry $CH_3CN$. The mixture was heated to reflux for two weeks. The solvent was removed under reduced pressure and the residue purified by chromatography on silica gel (eluent from $CHCl_3$ 100% to $CHCl_3$/MeOH = 80:20) to give 0.29 g of **1** (yield 48%) as a brown oil. $^{1}$H-NMR (δ DMSO-d6, 300 MHz) ppm: 8.09 (s, 1H, C=CH); 4.85-4.59 (m, 2H, $OCH_2C$=C); 4.50 (t, $^{3}J_{HH}$ = 5.1 Hz, 2H, $NNC\underline{H}_2CH_2O$); 4.24 (d, $^{3}J_{HH}$ = 7.8 Hz, 1H, H-1); 4.05 (s, 4H, OH); 3.80 (t, $^{3}J_{HH}$ = 5.1 Hz, 2H, $NNCH_2C\underline{H}_2O$); 3.75-3.65 (m, 3H, $OC\underline{H}_2CH_2NCH_3$, H-6[1H]); 3.55-3.42 (m, 9H, $OCH_2CH_2O$, H-6[1H]); 3.19-2.89 (m, 8H, H-2, H-3, H-4, H-5, $OCH_2C\underline{H}_2NC\underline{H}_2$); 2.66 (s, 3H, $NCH_3$); 1.67-1.52 (m, 2H, $CH_3NCH_2C\underline{H}_2$); 1.31-1.15 (m, 26H, $NCH_2CH_2[C\underline{H}_2]_{13}CH_3$); 0.83 (t, $^{3}J_{HH}$ = 6.6 Hz, 3H, $CH_2C\underline{H}_3$). $^{13}$C-NMR (δ DMSO-d6, 75 MHz) ppm: 145.40; 126.38; 103.91; 78.76; 78.50; 75.17; 71.88; 71.54; 71.44; 71.36; 70.54; 70.27; 63.27; 59.21; 58.09; 51.13; 43.98; 33.12; 30.84; 30.76; 30.52; 28.61; 28.29; 23.91; 15.76. Elemental analysis for C, H, N was within the theoretic value.

*2.4. Determination of critical micellar concentration (cmc) of GA **1***

Aqueous solutions (3 mL) of amphiphile **1** at concentrations between 0.1 μM and 1 mM 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 at 40°C under stirring for 12 h. Emission spectra of the solutions were acquired in the range 350-450 nm ($\lambda_{exc}$=335 nm) at 25°C. The *cmc* was defined as the concentration where the plot of intensity ratio of the third ($I_3$, 380 nm) and first ($I_1$, 370 nm) vibronic peaks of pyrene $I_3/I_1$ *versus* GA **1** concentration begins to raise.

*2.5 Determination of $pK_a$ value of amphiphile **1** in aggregative conditions*

Small aliquots of a HCl 1 mM aqueous solution were added to a 0.1 mM aqueous solution of amphiphile **1** (above its *cmc*) in saline buffer NaBr 0.390 mM. The $pK_a$ value of **1** was estimated by using the Gran's plot (after equivalence point):

$$10^{pH}V_a=K_a^{-1}(V_e-V_a)$$

where $V_a$ is the total added volume of acid and $V_e$ is the equivalence volume.

*2.6. Liposome preparation*

Aqueous dispersions of DMPC/**1** liposomes were prepared according to a reported procedure [10]. Lipid films were prepared on the inside wall of a round-bottom flask by evaporation of solutions containing the proper amount of DMPC and **1** (dissolved in $CHCl_3$) to obtain the desired molar percentage mixture. In the case of experiments relative to determination of the surface potential value (Ψ) of DMPC/**1** liposomes, heptadecyl-7-hydroxycoumarin (HC) (dissolved in THF) was added to the lipid solution to obtain a final 15 μM concentration. The obtained lipid films were kept overnight under reduced pressure (0.4 mbar); then a proper amount of PBS was added to obtain a lipid dispersion of the desired concentration. The aqueous suspensions were vortex-mixed and the obtained multilamellar vesicles (MLV) were subjected to DSC measurements at a 0.1 mg/μL (14.8 mM total lipids) concentration. For all the other experiments the MLV were freeze-thawed six times from liquid nitrogen temperature to 50 °C and were then extruded (10 times) through a 100 nm polycarbonate membrane.

*2.7 Determination of aggregate size by DLS*

DLS measurements were carried out at 25°C on aqueous solutions of amphiphile **1** (80 mM) and suspensions (1.25 mM total lipids in PBS) of DMPC/**1** liposomes at 95:5, 80:20, 70:30 molar ratio.

*2.8 Determination of thermotropic properties of liposomes*

DSC measurements were carried out on 30 μL of MLV aqueous dispersions at 0.1 mg/μL (148 mM total lipids) using aluminium pans. Two heating scans were recorded at the rate of 5°C $min^{-1}$ and two subsequent heating scans were recorded at the rate 1°C $min^{-1}$. Under the experimental

conditions, reproducible thermal recordings were obtained. Uncertainty on temperatures was determined to be 0.1°C.

*2.9 Determination of surface potential (Ψ) of liposomes*

An indirect method, described in literature and based on the pH-sensitive fluorophore HC [8-11], was exploited to determine Ψ of DMPC/**1** liposomes at molar ratios 95:5, 80:20 and 70:30 (total lipid concentration [DMPC]+[**1**]=5 mM) adding HC dissolved in THF to the lipid film to obtain a final concentration of 15 μM. Fluorescence measurements of liposome suspensions in PBS were performed by scanning the excitation wavelength between 300 and 400 nm, at the emission wavelength of 450 nm, varying the pH of solution between 2 and 12 by addition of concentrated aqueous NaOH or HCl. The extent of dissociation of liposomes including HC was monitored by the ratio of the excitation fluorescence intensities at 380 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 the Boltzmann equation:

$$\psi = -e^{-1}(pK_a^{charged} - pK_a^{neutral})k_BTln10$$

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 DMPC bilayer chosen as neutral reference.

*2.10 Agglutination of glucosylated liposomes by ConA monitored by OD measurements*

The variation of specific turbidity at 25°C of 1 mL samples of 0.83 mM DMPC/**1** (95:5, 80:20 and 70:30) liposomes in PBS upon the addition of ConA (0.33 mg/mL) was recorded over 500 minutes. Scans were carried out in a 1-cm quartz cell upon addition of ConA in PBS at 525 nm immediately after addition of ConA and every minute.

*2.11 Evaluation of release from DMPC/**1** liposomes as a function of pH*

Lipid films of DMPC and DMPC/**1** at 95:5, 80:20, 70:30 molar ratio were hydrated with 2 mL of a solution of pyr-m 0.1 mM in PBS to obtain 1 mM total lipid concentration. 150 μL of the extruded

suspension were added to 3 mL of a $5 \cdot 10^{-5}$ M solution of $FeCl_3$ in PBS at pH=7.4 or at pH 4.7 (the pH was adjusted by addition of small volumes of concentrated HCl before the beginning of the release test). The release of pyr-m was monitored at 30°C by following the decrease of the intensity of the first vibronic peak of pyr-m at 375 nm ($\lambda_{ex}$= 335 nm) as a function of time.

Rate release of pyr-m at pH 4.7 was evaluated by plotting the percentage of drug released from liposomes as a function of the square root of time according to Higuchi model. Release rate constant (*Kd*) were evaluated as the slope of the linear plot obtained.

## 3. Results

### *3.1 Characterization of GA **1***

Aqueous solutions of GA **1** at 10 mM and 4 °C are completely transparent and stable, thus indicating that GA **1** is characterized by low (<4 °C) Krafft point and Krafft temperature. The *cmc* of GA **1** was determined by a fluorescence method reported in the literature and resulted ($5.1 \pm 0.3) \cdot 10^{-6}$ M [12]. Above its *cmc* GA **1** forms micelles characterized by a hydrodynamic diameter ($d_h$) = 6 ± 0.8 nm as determined by DLS measurements. The application of the Gran's plot to the data obtained by the acid-base titration of GA **1** gave an apparent $pK_a = 3.7 \pm 0.2$.

### *3.2 Characterization of DMPC/**1** liposomes*

DLS measurements of DMPC/**1** liposomes at 95:5, 80:20 and 70:30 molar ratio showed that all the investigated formulations feature a stable monomodal distribution with a $d_h$ centered at ~100 nm (Table 1).

The thermograms relative to DMPC/**1** liposomes are reported in Fig. 1. The main transition temperature (Tm) is shifted to lower values with respect to the Tm of DMPC (Fig. 1) and the extent of the decrease depends on the amount of GA **1** in the formulation. Also ΔH values are lower if compared to ΔH of liposome composed of pure DMPC (Table 1). It can be observed that when the amount of GA **1** in the formulation is low the profile of the peak relative to the main transition is

very similar to that of pure DMPC liposomes. On the other hand, when the amount of GA **1** increases the peak in the thermograms are sensibly broader.

Finally, as reported in Table 1 similar positive values of Ψ° were estimated for all the DMPC/**1** formulations by means of the spectrofluorimetric titration.

*3.3 Interaction of DMPC/**1** liposomes with ConA*

DMPC/**1** liposomes at molar ratio 80:20 and 70:30 showed a neat increase of OD upon the addition of ConA, whereas no significant OD changes were detected in the case of the 95:5 formulation (Fig. 2). At molar ratio 80:20 vesicle agglutination occurs faster and to a higher extent with respect to the 70:30 formulation. Moreover, 80:20 liposome suspension showed a decrease of OD ~ 400 minutes after the addition of ConA clearly indicating precipitation of particles. DMPC liposomes (in the absence of glucosylated lipids) agglutinate in the presence of ConA over a longer range of time with respect to DMPC/**1** liposomes, and give rise to precipitation after 24 hours due to aspecific interactions.

*3.4 Evaluation of release from DMPC/**1** liposomes as a function of pH*

The capability of DMPC and DMPC/**1** liposomes to release an entrapped drug was evaluated at physiological pH (7.4) and acidic pH (4.7) by monitoring the decrease of the intensity of fluorescence of pyr-m, a fluorescent hydrophilic molecule used as drug model, upon dilution in PBS containing $FeCl_3$ as quencher. The intensity of the fluorescent signal is proportional only to pyr-m retained in the internal compartment of liposomes, because when the dye is released from the internal aqueous compartment of liposomes, its fluorescence is quenched by $FeCl_3$ dissolved in the bulk. At pH 7.4 DMPC and 95:5 DMPC/**1** liposomes release less than 10% of the entrapped dye over 3 h of incubation upon dilution, whereas in the case of 80:20 and 70:30 DMPC/**1** liposomes the amount of released dye reaches ~20% (data not shown). At pH=4.7 all the formulations show a higher release with respect to neutral conditions (Figure 3). However, it is evident that liposomes devoid of the glucosylated component release the lowest amount of dye (25% after 3 h), whereas all

the formulation containing GA **1** release a much higher amount (~50% after 3 h) of the entrapped dye.

The obtained *Kd* values are 2.26, 3.80, 4.42 and 5.13 % $min^{-1/2}$ for DMPC, DMPC/**1** 95:5, DMPC/**1** 80:20, DMPC/**1** 70:30 liposomes, respectively ($r^2$ were greater than 0.955, the graph from which *Kd* were evaluated are reported in Figure SI 2). It can be clearly noticed that, differently from what observed evaluating the amount of released pyr-m, *Kd* values increase as a function of the amount of GA present in the formulation.

## 4. Discussion

GA **1** is the pH-sensitive analogue of GA **2**, a quaternary ammonium surfactant that was shown able to interact with Con A but that can not be included in liposome formulations above 5 molar percentage because of its detergent effect [9]. Thus the tertiary ammine GA **1** was synthesized to have the possibility of increasing the amount of glucosylated component in the formulation. Further, because the protonation of the new lipid attributes to it a detergent effect, its presence in the formulation allows controlling drug release in acidic compartments such as lisosomes and/or tumour tissues. Moreover, its pH-sensitivity can facilitate the release of the entrapped drug to the target tissue.

As expected, GA **1** features a *cmc* ($5.4 \cdot 10^{-6}$ M) lower than the *cmc* of GA **2** ($1 \cdot 10^{-3}$ M) [9]. In fact, GA **1** is only partially protonated in micellar aggregates, therefore it has a behavior that is in between those of ionic and non-ionic surfactants that usually feature lower *cmc* values with respect to ionic surfactants. The low apparent $pK_a$ value of 3.7 obtained in the case of GA **1** micelles indicates that within the aggregates the amphiphile has less basic properties than expected for a tertiary ammine, *i.e.* in the micelle the protonation equilibrium shifts toward the deprotonated form, in agreement with data reported in the literature for other surfactants with tertiary amine head group

[14]. Literature reports show that the extent of the shift of $pK_a$ mainly depends on the dissociation extent of the surfactant in the aggregate [15], on the electrostatic interaction between the polar headgroups [16] and on a charge effect due to a dielectric discontinuity between the water environment and the interior of the micelle (aspect that influences transfer of charged and uncharged species from bulk water to the surface of a micelle) [17].

The absence of a net cationic charge on the headgroup of GA **1** reduces both detergent and disturbing effects on the lipid bilayer of liposomes with respect to its cationic analogue GA **2** [9]. In fact, DLS measurements show that phospholipid liposomes containing up to 30 molar % of GA **1** are stable and feature a hydrodynamic radius of ≈ 100 nm, in good agreement with the dimension imposed by the extrusion protocol, whereas 10 molar % of GA **2** partially solubilizes the lipid bilayer of phospholipid vesicles. This is an interesting result because it demonstrates that it is possible to decorate the surface of liposomes with a higher amount of targeting residues.

DSC measurements allowed investigating the influences of the presence of GA **1** on the lipid organization of DMPC liposome. Both the shift of Tm to lower values and the decrease of ΔH associated with the transition from gel to liquid-crystalline phase with respect DMPC liposomes (Fig. 1, Table 1) suggest that the packing of lipid in the bilayer is loosened by the presence of the synthetic amphiphile [18]. In the case of DMPC/**1** liposomes at 95:5 molar ratio a very low decrease of Tm and ΔH values are observed, indicating that the interaction of the alkyl chains are slightly reduced with respect to the mere DMPC whereas the interaction between polar headgroups is not affected by the presence of the GA **1** [19]. On the other hand, when the same amount of GA **2** was included in DMPC liposomes, though the decrease of Tm was the same, the effect on lipid packing was significantly different. In fact GA **2**, being scarcely miscible with DMPC, had an evident disturbing effect on the phospholipid bilayer indicated by the large range of temperature at which the transition occurs [9]. The increase of the amount of GA **1** in the formulation causes a significant reduction of lipid packing, interaction between polar headgroups and cooperativity of the transition. This behavior is rather different compared to that observed in other mixed systems containing a pH-

sensitive synthetic amphiphile [20]. Such a difference could be ascribed to the hygroscopic effect of the PEG spacer that, causing the removal of water from the hydration shell of lipid headgroups, affects the Tm value [21]. The weaker interactions of the alkyl chains of lipid membrane induce a reduction in the cooperativity of the transition as reflected by the width of the DSC transition peak. As a matter of fact, it has been previously observed that, in the same condition, a longer polyoxyethilenic spacer determines a decrease of cooperativity of the main transition [13].

$\Psi_o$ of DMPC/**1** liposomes was determined by the spetrofluorimetric titration of HC, a pH-sensitive probe that, when embedded in a lipid bilayer, locates in proximity of the headgroups. The fact that all the investigated formulations, independently from their composition, feature similar values of $\Psi_o$ indicates that GA **1**, as also observed in the micelles it forms, is not protonated even when included in phospholipid liposomes. Despite composed of uncharged and zwitterionic lipids, all the formulations show positive $\Psi_o$ values: this result can be ascribed to a different localization of the probe in DMPC/**1** liposomes with respect to liposomes of pure DMPC (chosen as neutral reference), as also observed in other systems [22]. The shift of HC in the bilayer is probably correlated with the loosening of bilayer packing induced by the addition of GA **1** as inferred from DSC experiments.

We also investigated the targeting properties of DMPC/**1** liposomes by agglutination assay evaluating their ability to interact with ConA, a tetrameric protein that, thanks to the presence of multiple binding sites for glucose, binds to more than one glycosylated liposome leading to aggregation of vesicles. The absence of interaction with ConA observed in the case of DMPC/**1** liposomes 95:5 cannot be ascribed to the low density of glucose moieties on vesicles surface because liposomes containing the same percentage of GA **2** in the same experimental conditions showed a great ability to interact with ConA [9]. The different behaviour of DMPC/**1** and DMPC/**2** liposomes is probably related to a different exposure of the glucose residue to the bulk due to the different organization of the lipid bilayer, as suggested by DSC experiments. Agglutination was observed upon the addition of ConA to 80:20 and 70:30 DMPC/**1** liposome suspensions. The minor and slower OD increase observed in the case of 70:30 with respect to 80:20 molar ratio indicates

that in the presence of a higher amount of GA **1** agglutination yields smaller aggregates. Evidently, the increase of the sugar moieties concentration on the liposome surface suppresses liposome crosslinking upon addition of ConA, due probably to a preferential intraliposome partial saturation of the protein binding sites. Literature reports confirm the dependence of the binding efficiency of carbohydrates with lectins on surface density of sugar moieties [23]. It must be stressed that under agglutination conditions the fraction of sugar moieties accessible to ConA is always larger than the protein binding sites ([GL**1**]/[ConA] binding sites being ≈ 2 and 3.5 for DMPC/**1** 80:20 and 70:30, respectively). This means that the binding sites of ConA are probably saturated in all the investigated samples and an increase of GA**1** fraction although determining an increase of sugar moieties does not promote a sensitive increment of the ConA sugar binding degree.

Finally, the pH-sensitivity of the investigated glucosylated formulations was assessed by fluorescence experiments comparing the release of a hydrophilic molecule, pyr-m, at neutral (7.4) and acidic pH (4.7), the latter being typical of endosomal compartment and of certain tumour cells [3]. At pH=7.4 DMPC/**1** liposomes at 95:5 molar ratio show the same modest capability of DMPC liposomes to release pyr-m, confirming that the presence of the neutral GA at low percentage does not affect packing. When the percentage of **1** in the formulation raises the release of pyr-m from the internal aqueous compartment is twofold higher indicating a loose packing of lipid molecules, in good agreement with results obtained by DSC experiments. At acidic pH, the release of the drug from all formulations containing the GA, independently of its amount, is significantly enhanced and is sensibly higher with respect to the release observed from DMPC liposomes. The absence of significant differences by increasing the GA content was observed also in the case of other pH-sensitive pegylated liposomes [24] and can probably be ascribed to the increased sterical stability conferred to liposomes by high amount of PEG-lipids. Drug release rate is an important parameter that can greatly affect the therapeutic efficacy of liposome formulation [25]. The fact that the plot from which the *Kd* values were evaluated are linear indicates that Higuchi's model was a good fitting model and that the release of pyr-m from the investigated liposomes was driven mainly by a

diffusion-controlled mechanism [26]. The obtained *Kd* values are in good agreement with those reported in literature investigating drug release profile from pegylated liposomes *in vitro* [27]. Differently from what observed evaluating the amount of drug released, the release rate seems to be strictly dependent on the amount of GA present in the formulation, thus suggesting that their efficacy as drug delivery system could be greatly affected by the presence of the glucosylated pH sensitive component.

As a whole, these results indicate that the new pH-sensitive liposomes have a good potential as delivery systems in which the release of the drug is triggered by pH sensitivity.

## 4 Conclusions

Recent efforts in liposome research are aimed at developing multifunctional formulations to increase their specificity to target tissue while controlling drug release thus improving their efficacy as drug delivery systems. We synthesized and characterized a novel pH-sensitive GA **1** and included it in DMPC liposomes at different molar ratios. The results of our investigations indicate that at physiological pH the new amphiphile is present in its deprotonated form both when self assembled, as pure component, in micelles and when included in DMPC liposomes. The comparison of the properties of DMPC/GA **1** liposomes with those containing its cationic analogue GA **2**, previously described, points out that the presence of the neutral amphiphile has a modest detergent effect on the lipid bilayer of phospholipid liposomes thus allowing to obtain stable formulations containing up to 30% of **1**. This represents a good result for targeting. Moreover, acidic pH induced a significant increase of drug release from all DMPC/**1** liposomes investigated. These features together with the targeting ability, make these multifunctional pH-sensitive glucosylated particles good candidates as drug delivery systems.

**Corresponding Author**

* E-mail: giovanna.mancini@uniroma1.it

**Notes**

The authors declare no competing financial interest.

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript

**Acknowledgements**

We acknowledge technical and administrative support by Mrs. Stefania Tarquini and Mr. Marco Pastore and the financial support of PRIN project Multinanoita (project number 2010JMAZML) and of the FIRB project 2012 RBFR12BGHO.

**References**

[1] P. P. Deshpande, S. Biswas, V. P. Torchilin. Current Trends in the Use of Liposomes For Tumor Targeting, Nanomedicine 8(9) (2013) 1509-1528.

[2] X. Liu, G Huang. Formation Strategies, Mechanism of Intracellular Delivery and Potential Clinical Applications of pH-Sensitive Liposomes, Asian J Pharm Sci. 8 (2013) 319-328.

[3] H. Karanth, R. S. R. Murthy. pH-Sensitive Liposomes-Principle and Application in Cancer Therapy, J. Pharm. Pharmacol. 59 (2007) 469-483.

[4] J. Huwyler, J. Drewe, S. Krähenbühl. Tumor Targeting Using Liposomal Antineoplastic Drugs, Int. J. Nanomed. 3(1) (2008) 21–29.

[5] V. P. Torchilin. Recent Advances With Liposomes As Pharmaceutical Carriers, Nat. Rev. Drug Discov. 4 (2005) 145-160.

[6] S. Nakahara, A. Raz. Biological Modulation by Lectins and Their Ligands in Tumor Progression and Metastasis, Anticancer Agents Med. Chem. 1 (2008) 22-36.

[7] M. Hill, D. Mazal, V. A. Biron, L. Pereira, L. Ubillos, E. Berriel, H. Ahmed, T. Freire, M. Rondán, G. R. Vasta, F. Liu, M. M. Iglesias, E. Osinaga, A Novel Clinically Relevant Animal Model for Studying Galectin-3 and its Ligands During Colon Carcinogenesis, J. Histochem. Cytochem. 58 (2010) 553-565.

[8] R. Lotan, A. Raz. Lectins in Cancer Cells, Ann. N Y Acad. Sci. 551 (1988) 385-398.

[9] A. Mauceri, S. Borocci, L. Galantini, L. Giansanti, G. Mancini, A. Martino, L. Salvati Manni, C. Sperduto, Recognition of Concanavalin A by Cationic Glucosylated Liposomes, Langmuir 30 (2014) 11301-11306.

[10] M. J. Hope, M. B. Bally, G. Webb, P. R. Cullis, Production of Large Unilamellar Vesicles by a Rapid Extrusion Procedure. Characterization of Size Distribution, Trapped Volume and Ability to Maintain a Membrane Potential, Biochim. Biophys. Acta 812 (1985) 55-65.

[11] N. J. Zuidam, Y. Barenholz, Electrostatic Parameters of Cationic Liposomes Commonly Used for Gene Delivery as Determined by 4-Heptadecyl-7-Hydroxycoumarin, Biochim. Biophys. Acta - Biomembranes 1329 (1997) 211-222.

[12] K. Kalyanasundaram, J. K. Thomas, Environmental Effects on Vibronic Band Intensities in Pyrene Monomer Fluorescence And Their Application in Studies of Micellar Systems, J. Am. Chem. Soc. 99 (7) (1977) 2039-2044.

[13] A. Mauceri, A. Fracassi, M. D'Abramo, S. Borocci, L. Giansanti, A. Piozzi, L. Galantini, A. Martino,V. D'Aiuto, G. Mancini, Role of the Hydrophilic Spacer of Glucosylated Amphiphiles Included in Liposome Formulations in the Recognition of Concanavalin A, Colloids Surf. B Biointerfaces 136 (2015) 232-239.

[14] A. Mezei, L. Pérez, A. Pinazo, F. Comelles, M. R. Infante, R. Pons, Self Assembly of pH-Sensitive Cationic Lysine Based Surfactants, Langmuir 28 (2012) 16761-16771.

[15] A. Goldsipe, D. Blankschtein, Titration of Mixed Micelles Containing a pH-Sensitive Surfactant and Conventional (pH-Insensitive) Surfactants: A Regular Solution Theory Modeling Approach, Langmuir 22 (2006) 9894-9904.

[16] A. Goldsipe, D. Blankschtein, Molecular-Thermodynamic Theory of Micellization of pH-Sensitive Surfactants. Langmuir 22 (2006) 3547-3559.

[17] O. Söderman, B. Jönsson, G. Olofsson, Titration of Fatty Acids Solubilized in Cationic and Anionic Micelles. Calorimetry and Thermodynamic Modeling, J. Phys. Chem. B 110 (2006) 3288-3293.

[18] R. L. Biltonen, D. Lichtenberg, The Use of Differential Scanning Calorimetry as a Tool to Characterize Liposome Preparations, Chem. Phys. Lipids 64 (1993) 129-142.

[19] C. Huang, S. Li, Calorimetric and Molecular Mechanics Studies of the Thermotropic Phase Behavior of Membrane Phospholipids, Biochim. Biophys. Acta, 1422 (1999) 273-307.

[20] Y. Barenholz, C. Bombelli, M. G. Bonicelli, P. di Profio, L. Giansanti, G. Mancini, F. Pascale, Influence of Lipid Composition on the Thermotropic Behavior and Size Distribution of Mixed Cationic Liposomes. J. Colloid Interface Sci. 356 (2011) 46-53.

[21] A. Blume, P. Garidel, in Richard B. Kemp (Eds), Handbook of Thermal Analysis and Calorimetry: From Macromolecules to Man, Elsevier, Amsterdam, 1999, Chapter 3, Lipid Model Membranes and Biomembranes, 121-123.

[22] C. Giuliani, B. Altieri, C. Bombelli, L. Galantini, G. Mancini, A. Stringaro, Remote Loading of Aloe Emodin in Gemini-Based Cationic Liposomes, Langmuir 31 (2015) 76-82.

[23] Y. Chen, M. S. Lord, A. Piloni, M. H. Stenzel. Correlation Between Molecular Weight and Branch Structure of Glycopolymers Stars and Their Binding to Lectins, Macromolecules 48 (2015) 346-357.

[24] V. A. Slepushkin, S. Simões, P. Dazini, M. S. Newman, L. S. Guo, M. C. Pedroso de Lima, N. Düzgünes. Sterically Stabilized pH-sensitive Liposomes, J. Biol. Chem. 272(4) (1997) 2382-2388.

[25] G. J. R. Charrois, T. M. Allen. Drug Release Rate Influences The Pharmacokinetics, Biodistribution, Therapeutic Activity, And Toxicity of Pegylated Liposomal Doxorubicin Formulations in Murine Breast Cancer, Biochim. Biophys. Acta Biomembranes, 1663(1-2) (2004) 167-177.

[26] M. M. Nounou, L. K. El-Khordagui, N. A. Khalafallah, S. A. Khalil. *In Vitro* Release of Hydrophilic And Hydrophobic Drugs From Liposomal Dispersions And Gels, Acta Pharm. 56 (2006) 311-324.

[27] A. Hioki, A. Wakasugi, K. Kawano, Y. Hattori, Y. Maitani. Development of an *in Vitro* Drug Release Assay of PEGylated Liposome Using Bovine Serum Albumin and High Temperature, Biol. Pharm. Bull. 33(9) (2010) 1466-1470.

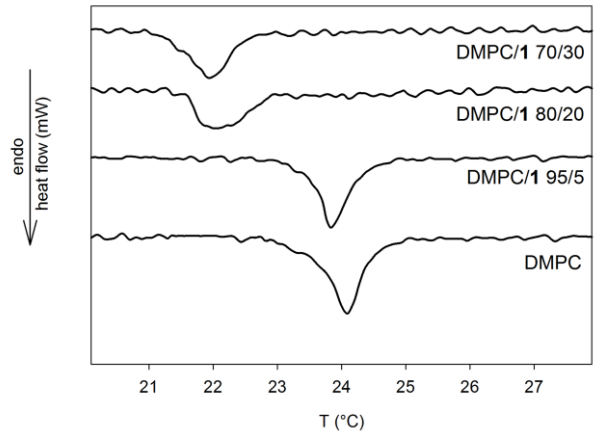



**Fig 1.** Thermograms relative to DMPC/**1** MLVs (at different molar ratios) organized according to increasing percentages of GA **1**, the first thermogram from the bottom being relative to vesicles formulated only with DMPC. Scan rate is 1 °C/min.

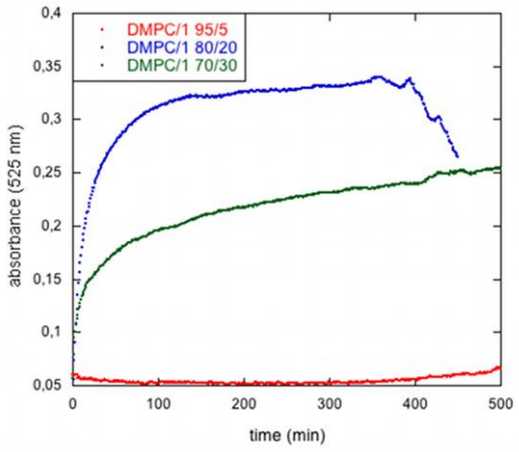



**Fig 2.** Agglutination of DMPC/**1** vesicles at 95/5 (red trace), 80/20 (blue trace) and 70/30 (green trace) molar ratio in PBS upon the addition of ConA monitored by optical density. ConA concentration 0.33 mg/mL; total lipids concentration 0.83 mM.

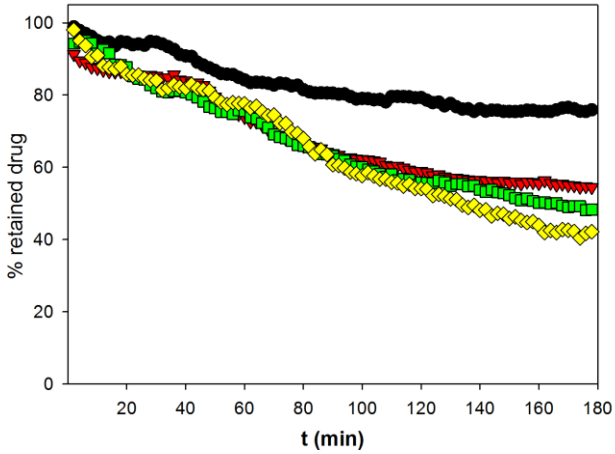
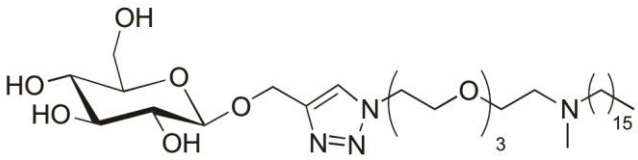
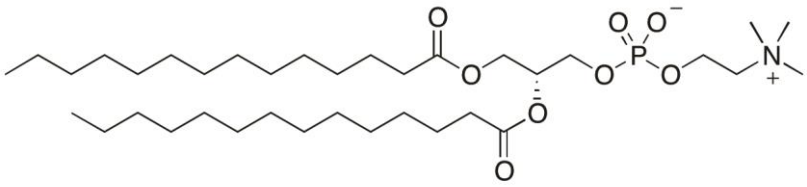



**Fig 3.** Percentage of 1-pyrenemethanol retained by DMPC/**1** vesicles at 100/0 (black circles), 95/5 (red circles), 80/20 (green down triangle) and 70/30 (yellow up triangle) molar ratio in PBS at pH 4.7 as a function of time. The reported data represent the average values of at least three different and independent experiments of each sample. Error in determination is within 5% (see Figure SI 1).

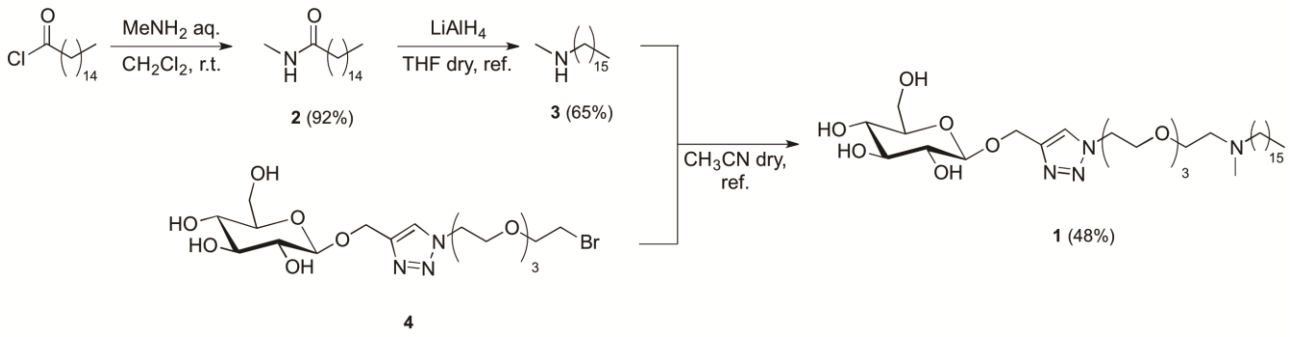



**1**




**DMPC**

**Chart 1.** Liposome components.




**Scheme 1.** Synthetic scheme for the synthesis of GA **1**

**Table 1.** Thermodynamic parameters of DMPC/**1** MLV and SUV. Temperature and ΔH values were determined with an accuracy of ±0.1°C and ±0.5 kJ/mol, respectively.

| DMPC/**1** | Tm (°C) | ΔH (kJ/mol) | $d_h$ (nm) | $\Psi_o$ (mV) |
|---|---|---|---|---|
| 95/5 | 23.8 | 20.8 | 96 ± 8 | 26 ± 6 |
| 80/20 | 22.0 | 25.8 | 84 ± 5 | 29 ± 5 |
| 70/30 | 21.9 | 23.2 | 108 ± 16 | 34 ± 5 |

Tm of DMPC liposomes: 24.1°C; ΔH of DMPC liposomes: 28.9 kJ/mol (data from ref [13~~0~~]).