

Electronic Supplementary Information

'One-pot' sequential enzymatic modification of synthetic glycolipids in vesicle membranes

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S1. Chemical provision and instrumentation

Chemical reagents and solvents used to perform the experiments were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK), Fisher Scientific UK Ltd and Alfa Aesar unless otherwise stated. The biochemical reagents fetuin, 3-sialyllactose and $MnCl_2$ solution were supplied by Sigma-Aldrich Co. Ltd. The phospholipids DMPC and DPPC were provided by Avanti Polar Lipids.

Phosphate buffered saline (PBS), cell culture media (EMEM), L-glutamine, non-essential amino acids, penicillin, streptomycin, accutase, foetal bovine serum (FBS), bovine serum albumin (BSA), well plates, tissue culture flasks, paraformaldehyde and DAPI were all obtained from Sigma-Aldrich Co. Ltd. AlexaFluor 488 Phalloidin was obtained from ThermoFisher Scientific Inc., USA. HepG2 cells were obtained from ECACC (*via* Sigma-Aldrich Co. Ltd.).

Enzymes were expressed and purified according to published procedures described by Flitsch *et al.*^{1,2} Lipid **1** was synthesised to literature procedures and gave satisfactory analytical data that was consistent with that previously reported.²

Liposomes were synthesised after first vortex mixing with a Vortex Genie 2 (Scientific Industries Inc.), followed by extrusion through a LiposoFast basic extruder, fitted with 800 nm polycarbonate membranes supplied by Avestin. Liposome analysis was performed using several different techniques: UV-visible spectroscopy was performed on a Jasco V-660 spectrophotometer and fluorescence spectroscopy was performed on a Perkin Elmer LS55 luminescence spectrometer. Samples assessed by Dynamic Light Scattering (DLS) were measured in HPLC grade water at 25 °C on a Malvern Zetasizer Nano S (He-Ne laser 633 nm) instrument.

Enzymatic glycosylation reaction progress was monitored by high-performance liquid chromatography (HPLC) using an Agilent 1200 series LC system with G1315B diode array detector and Agilent 1100 MSD Ion Trap (MS detector), using Machery Nagel C18 Nucleosil column (250 × 4.4 mm, 5 μM).

Stained cell samples were imaged using Leica TCS SP5 confocal fluorescence microscopy using the oil objective (63 ×). The fluorescence of each sample was analysed on a BD LSR Fortessa flow cytometer using the yellow-green laser ($\lambda = 561$ nm) for the excitation of the rhodamine fluorophore in the vesicle membranes.

S2. Experimental procedures for vesicle formation and characterisation

The required lipid mixture (1.80 mmol total lipid; DMPC 1.22 mg, DPPC 1.32 mg, 1:1 DMPC/cholesterol 0.61 mg/0.35 mg) and lipid **1** (0.22 mg, 0.20 μmol) were dissolved in spectroscopic grade $CHCl_3$ (1 mL) and the solvent removed *in vacuo* to generate a thin film. The lipid film was further dried under reduced pressure to remove trace solvent, then rehydrated with MES buffer (1 mL, 50 mM, titrated with NaOH to give pH 7 at 25 °C). The buffer solution was vortex mixed for 10-20 minutes, until the lipids were dispersed. Large unilamellar vesicles were formed by repeated extrusion (19 ×) of the resulting suspension through a polycarbonate membrane (800 nm pore size) above the lipid T_m .

The extent to which lipid **1** was incorporated into vesicle membranes was measured using published procedures.² Samples of diluted DMPC, DMPC/cholesterol and DPPC vesicles (200 μ L, 20 μ M solution in MES) doped with the starting loading of 10 % mol/mol of **1** were formed; the UV-visible spectrum of each mixture measured both before and after extrusion. The samples were purified by gel permeation chromatography (GPC). Unbound **1** was removed from the vesicular suspension using PD-10 gel permeation desalting columns. The GPC columns were equilibrated with MES buffer (4 \times 20 mL) before the lipid suspension was added to the column (200 μ L made up to 2.5 mL with 2.3 mL MES). Once the sample had fully eluted onto the column, MES buffer (3.5 mL) was added to the top and the UV-visible spectra of the eluent (3.5 mL, 11.4 mM lipid) was measured. The amount of lipid **1** incorporated into the vesicles was determined by calculating the difference in peak height (using the maximum UV absorption of pyrene at $\lambda_{\text{max}} = 346$ nm) of **1** before extrusion, after extrusion and after GPC (after correction for dilution).

S3. Experimental procedures for enzymatic transformation of 1 and 4

A suspension of phospholipid vesicles doped with **1** (100 μ L, 2.0 mM total lipid; **1**-DMPC, 170 μ M **1**; **1**-DMPC/chol, 128 μ M **1**) were added to a reduced volume mass spectrometry vial. This suspension was mixed with UDP-Gal (5 μ L, 10 mM), MnCl₂ (1 μ L, 1 M solution), β 4Gal-T1 (5 μ L, 0.42 mg/mL), TcTs (5 μ L, \sim 3.91 μ M) and 3-sialyllactose (5 μ L, 6 mM). The mixture was incubated at room temperature (22 $^{\circ}$ C) for 7 h.

In analogous solution phase experiments PNP-GlcNAc **4** (100 μ L, 200 μ M **4** in 50 mM MES buffer) was reacted added to a reduced volume mass spectrometry vial, then mixed with UDP-Gal (5 μ L, 10 mM), MnCl₂ (1 μ L, 1 M solution), β 4Gal-T1 (5 μ L, 0.42 mg/mL), TcTs (5 μ L, \sim 3.91 μ M) and 3-sialyllactose (5 μ L, 6 mM). The mixture was incubated at room temperature (22 $^{\circ}$ C) for 1.5 h.

S4. Reversed phase HPLC procedures and data analysis

Reaction progress for **1** in vesicles was monitored by reversed phase analytical HPLC. Sample aliquots (5 μ L) from the reaction mixture were removed and analysed every 0.5 h for 7 h. A solvent gradient (35-80 % MeCN in H₂O, 0.5 mL/min flow rate, 10 μ L MeOH needle wash) was used to elute each aliquot. The HPLC DAD module was set to record pyrene absorbance at 346 nm.

For the solution phase experiments with **4**, sample aliquots (5 μ L) were analysed by reverse phase analytical HPLC at shorter interval time points (0, 5, 15, 25, 35, 45, 60 and 90 minutes). An isocratic solvent method (15% MeCN in H₂O, 0.5 mL/min flow rate, 10 μ L MeOH needle wash) was used to elute the samples with HPLC DAD module set to record absorbance at 300 nm.

Peak areas were used to perform data analysis. Each constituent was calculated as a proportion of the total population of compounds that had some absorbance at the wavelength of interest.

S5. Fetuin-catalysed hydrolysis of PNP-GlcNAc 4

A solution of PNP-GlcNAc (100 μ L, 200 μ M in 50 mM MES buffer) was added to a reduced volume mass spectrometry vial with UDP-Gal (5 μ L, 10 mM), MnCl_2 (1 μ L, 1 M solution), β 4Gal-T1 (5 μ L of 0.42 mg/mL), TcTs (5 μ L, \sim 3.91 μ M) and fetuin (5 mg, estimated 6 mM, see Wilbrink *et al.*).³ The mixture was incubated at room temperature (22 $^{\circ}$ C) for 10 h. On addition of fetuin the colourless solution turned yellow. Reaction progress was monitored (aliquots of 5 μ L taken every hour) by reversed phase analytical HPLC on a Machery Nagel C18 column (250 \times 4.6 mm, 5 μ m). This reverse phase analytical HPLC trace of the reaction mixture showed a single peak with a retention time of around 4 minutes, which we ascribe to the formation of *p*-nitrophenol, and small peak at the retention time of the starting material (21 minutes, Figure S1). The reaction was monitored over a 10 h incubation period.

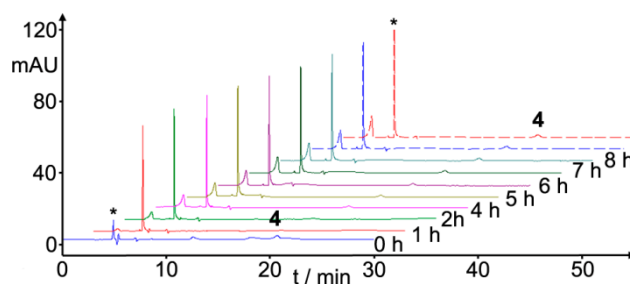


Figure S1. Reverse phase HPLC traces showing fetuin-mediated hydrolysis of PNP-GlcNAc **4** over 10 hours. Asterisk indicates a new and growing peak, ascribed to the formation of *p*-nitrophenol from the hydrolysis of **4**.

S6. LCMS procedures and data

Reversed phase analytical HPLC was performed on aliquots (5 μL) taken from the reaction mixtures for sequential enzymatic transformation of **1** and **4**. A solvent gradient (35-80 % MeCN in H_2O , 0.5 mL/min flow rate, 10 μL MeOH needle wash) was used for reaction of **1** in membranes and an isocratic method for **4** in solution (see Section S4). HPLC was fitted with an in-line mass spectrometer (reflector negative mode) set to display m/z ratios of compounds detected at selected wavelengths (346 nm for pyrene containing compounds and 300 nm for 4-nitrophenyl containing compounds).

Three of the peaks in the HPLC trace for the transformation of **1** gave positive ions with m/z values consistent with each of the respective products (Figure S2). A decrease in retention time occurred as saccharide units were added to the lipid **1**.

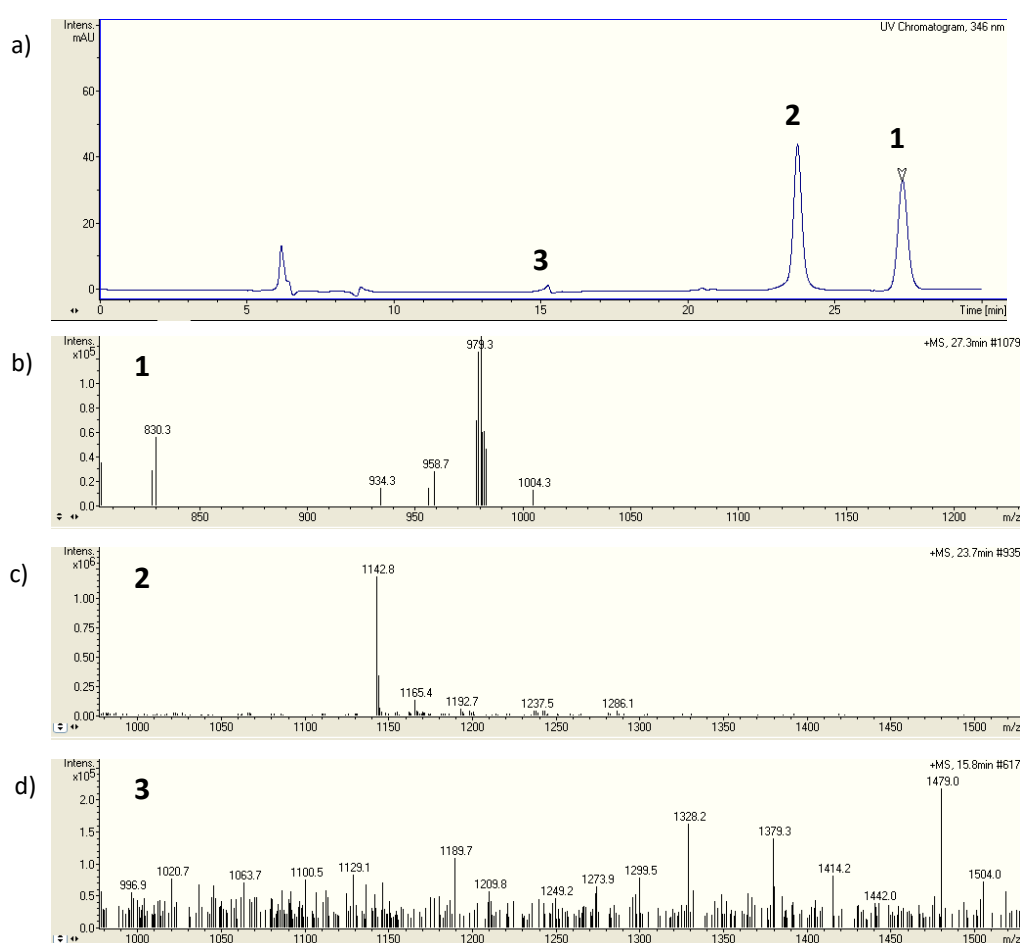


Figure S2. Partial LCMS spectra of 8.5% mol/mol **1** in DMPC vesicles after galactosylation by $\beta 4\text{Gal-T1}$ and sialylation by TcTS. (a) HPLC trace. (b-d) MS spectra from peaks due to (b) **1**; (c) **2**; (d) **3**. Identified ions: **1**, m/z of 979.3 ($[\text{M}]^+$); **2**, m/z of 1142.8 ($[\text{M}]^+$); **3**, m/z of 1479.0 ($[\text{M} + 2\text{Na}]^+$).

S7. 'One-pot' sequential enzymatic transformation of 1-DPPC vesicles.

The procedures described in Sections S3, S4 and S6 were followed, but using 1-DPPC vesicles in the place of 1-DMPC vesicles. Although the data obtained was analogous to that in the other lipid mixtures (Figure S3), the quality of the data obtained after HPLC analysis was poorer, with greater variability between samples. Nonetheless, similar trends can be observed as found with the other vesicle mixtures (Figure S4), with lower reactivity at the vesicle interface. However, the production of the sialylated derivative was greater and there was less accumulation of the galactosylation intermediate.

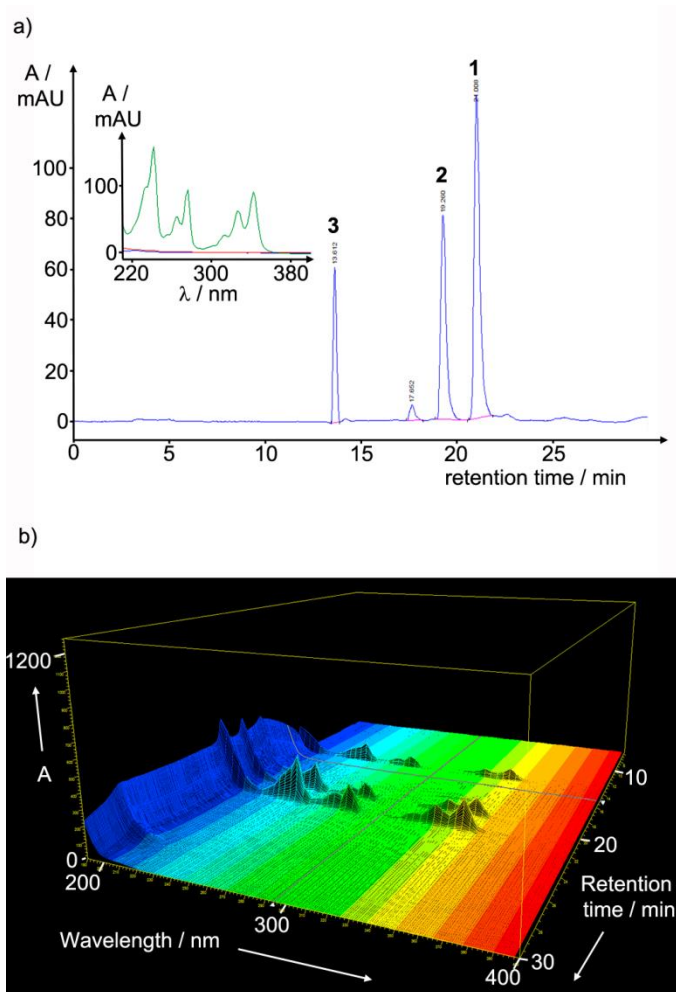


Figure S3. Analytical reverse phase HPLC trace for **1** embedded in DPPC vesicles (8.5 % mol/mol, see reference [4]) after reaction with β 4Gal-T1 and TcTS after 1 h, showing peaks corresponding to **1**, **2**, and **3**. HPLC DAD monitoring absorbance at 346 nm. Inset: UV-visible absorption spectra take at retention times of 13.424 (blue trace), 13.611 (green trace) and 13.884 min (red trace); characteristic pyrene absorbance observed at 13.611 min. b) Three dimensional plot of time, absorbance and wavelength showing characteristic pyrene absorbance for each eluted peak.

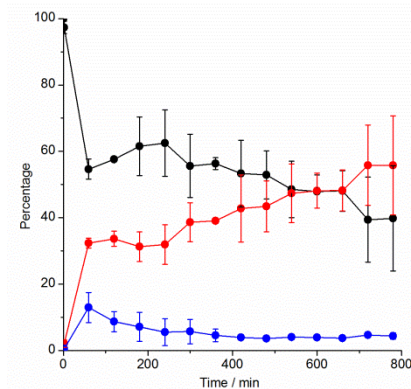


Figure S4. Plot showing the change over time in the relative proportions of GlcNAc (black, ●), LacNAc (blue, ●) and Neu5Ac (red, ●) terminated products upon ‘one-pot’ transformation by β 4Gal-T1 and TcTS acting on **1** clustered into microdomains in the membranes of DPPC vesicles at 8.5 % mol/mol.⁴ Error bars show standard deviations from three measurements.

S8. Preliminary cell-vesicle experiments and confocal fluorescence microscopy (CFM).

In order to evaluate the recognition of functionalised liposomes by cells, a cellular uptake experiment was performed using a hepatocellular carcinoma cell line (HepG2). To optimise the best cell culture conditions (time-points and cell density) and liposome concentrations, these preliminary studies were carried out using only two types of vesicle, unmodified (**1**-DMPC, GlcNAc-coat) and transformed (**1**-DMPC+ β 4Gal-T1, LacNAc coat). The variables assessed in the cell culture conditions were:

- (a) two cell densities (1×10^4 and 2.7×10^4 cells/mL) were tested
- (b) different ratios of adhesion/incubation time (2 h cell adhesion/24 h cell uptake and 24 h cell adhesion/3 h cell uptake),
- (c) three liposome concentrations (5.3, 13.3 and 26.3 μ g/mL).

At the end of each experiment, samples were processed for F-actin and DNA staining, then imaged by confocal microscopy. In general, the confocal fluorescence microscopy images after either time-points (24 h adh. with 3 h inc. or 2 h adh. with 24 h inc.) show preferential uptake of β 4Gal-T1 transformed **1**-DMPC vesicles by HepG2 cells (Figure S5). This is more evident in the shorter uptake time (24 h adhesion, 3 h liposome uptake). At a longer liposome uptake time (24 h liposome uptake, albeit at the shorter 2 h adhesion) the differences between **1**-DMPC (GlcNAc) and **1**-DMPC + GalT1 were not so evident. These data suggest that because internalisation is non-reversible, at longer time-points the differences between different coatings may appear to be less, especially as cells seem to internalize all types of saccharide-coated vesicles to some extent.

These CFM experiments were also used to optimise conditions for later CFM (using all vesicle types) and flow cytometry on the cell/vesicle mixtures:

- (a) Cell density was varied, which showed the highest cell density (2.7×10^4 cells/mL) produced cell clusters, but the formation of these clusters did not interfere with the vesicle uptake (data not

shown). Based on these observations, and the fact that HepG2 cells grew better when in higher densities, the concentration of 2.7×10^4 cells/mL was selected for further CFM and flow cytometry studies.

(b) Cell adhesion times of 2 h and 24 h were both assessed in these initial CFM experiments. The latter seemed to be better suited for analysing this type of cell under this experimental protocol, as it allowed the cells to adhere and spread on the surface of the plates, as well as making cell imaging more straightforward. Therefore the 24 h adhesion time was selected for further CFM and flow cytometry studies. Changing the incubation time of the vesicles with the cells was also assessed; given the observed changes over time in these CFM experiments, both 3 h and 24 h were selected for further study.

(c) The images obtained in these preliminary experiments (Figure S5) did not permit the best vesicle concentrations for probing difference in cell uptake to be unambiguously determined, so both the smaller ($5.3 \mu\text{g/mL}$) and higher ($26.7 \mu\text{g/mL}$) vesicle concentrations were selected for further flow cytometric analysis.

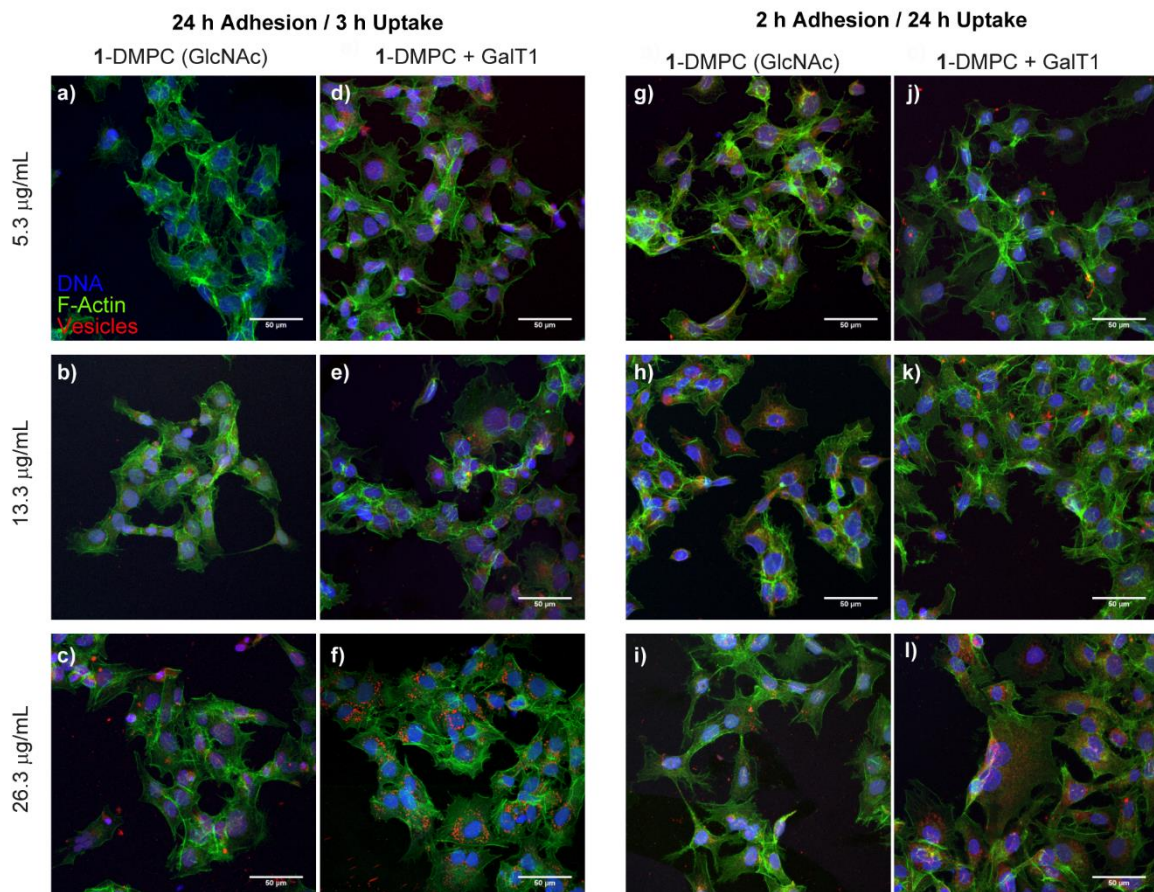


Figure S5. Cellular uptake of 1-DMPC (GlcNAc) and 1-DMPC + GalT1 vesicles by HepG2 cell line (2.7×10^4 cells/mL). Different conditions were evaluated, namely: Analysis time point - 24 h cell adhesion and 3 h liposome uptake (a-f); 2 h cell adhesion and 24 h liposome uptake (g-l). Liposome concentration: $5.3 \mu\text{g/mL}$ (a, d, g, j), $13.3 \mu\text{g/mL}$ (b, e, h, k) and $26.7 \mu\text{g/mL}$ (c, f, i, l). Samples were stained with DAPI (DNA), 488 phalloidin (F-actin) and rhodamine (vesicles) and imaged under confocal fluorescence microscopy using an oil 63 \times objective. Scale bar = 50 μm .

S9. Final CFM conditions and flow cytometry.

After optimising the conditions for evaluation of vesicle uptake by HepG2 cells, four liposome formulations were synthesised, namely DMPC only, 1-DMPC (GlcNAc coating), 1-DMPC+ β 4Gal-T1 mixture (LacNAc coating) and 1-DMPC+ β 4Gal-T1+TcTS mixture (some sialic acid coating). The different vesicle formulations were tested at two concentrations: 5.3 μ g/mL and 26.7 μ g/mL, and at two incubation time points: 3 h and 24 h. The adhesion time was fixed at 24 h. At the end of each experiment, samples were processed for F-actin and DNA staining, then imaged by confocal fluorescence microscopy (images shown in Figure 4 of the main text). In order to quantify the number of liposomes that were internalised by HepG2 cells, plates cultured in parallel (under the same conditions as those used for confocal fluorescence microscopy) were processed for flow cytometry analysis.

HepG2 cells were seeded in 6-well plates and incubated for 24 h to allow adhesion of cells. The media was then removed and fresh media containing vesicles (liposomes) was added. After 3 h or 24 h of incubation with vesicles, cells were fixed in 3.7% (w/v) paraformaldehyde (PFA) solution diluted 1:3 in PBS (20 min, 37°C) and centrifuged (1100 rpm, 3 min, 4°C). Cells were washed with FACS buffer (2% FBS and 0.1% azide in PBS) and centrifuged to remove the buffer.

The fluorescence of each sample was analysed on a BD LSRFortessa flow cytometer, with the yellow-green laser used for the excitation of the fluorophore. Cell debris and dead cells were excluded from the analysis based on electronic gates using forward scatter (size) and side scatters (cell complexity) criteria. All the settings were determined at the start of the experiment using HepG2 cells that were not cultured with vesicles (liposomes) as the negative control (Figure S6).

A total of 10,000 events were recorded for each sample that was studied by flow cytometry. The flow cytometry results are presented as a box-plot chart (10th percentile, first quartile, median, third quartile and 90th percentile), with the mean represented as “•” (Figure S7). For statistical analysis, the parametric test one way ANOVA was used for the comparison between groups. The assumption of the homogeneity of variances was assessed using Levene’s test. The statistical difference between datasets was found to violate the assumption of the homogeneity of variances; therefore, the Games-Howell test was used. A p value <0.05 was considered statistically significant.

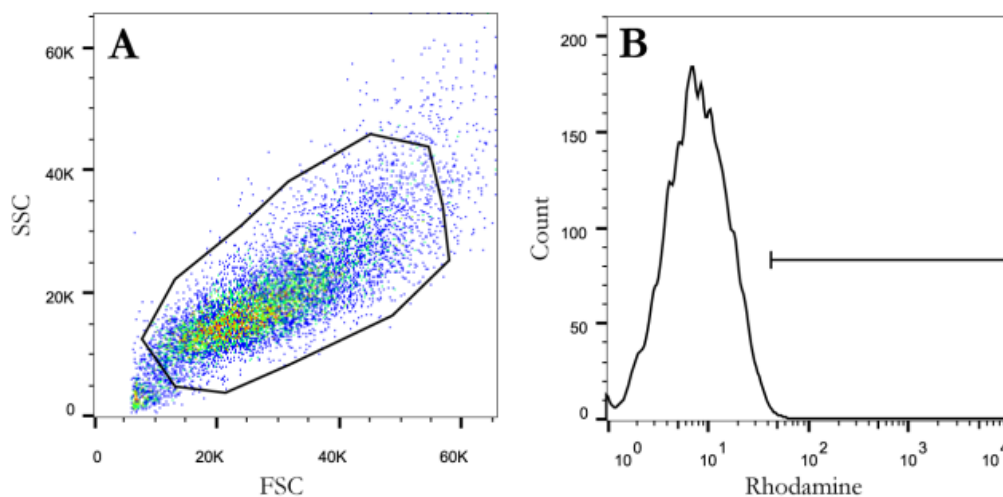


Figure S6. HepG2 cells that were not cultured with liposomes were used to establish the parameters of detection of the fluorescence. Cell debris and dead cells were excluded based on gates using forward scatter (size) and side scatters (cell complexity) criteria. First, the cell population was demarcated (excluding debris) (A) and then the autofluorescence of these cells was quantified (B).

The flow cytometry results (Figure S7) show that both an increase in incubation time and an increase in vesicle concentration led to a corresponding increase in vesicle internalisation by cells.

At the lowest vesicle concentration (Figure S7a), both time points (3 h and 24 h) showed that uncoated DMPC vesicles exhibited significantly lower uptake as compared with the other liposome formulation. At this vesicle concentration, the FACS data for the **1-DMPC+ β 4Gal-T1** mixture (LacNAc coating) correlated with the CFM images, showing that the **1-DMPC+ β 4Gal-T1** mixture had a best uptake at 3 h. However upon extending the incubation time to 24 h at this vesicle concentration, both types of enzymatically transformed vesicles, **1-DMPC+ β 4Gal-T1** (LacNAc coating) and **1-DMPC+ β 4Gal-T1+TcTS** (some sialic acid coating), showed similar values of uptake.

At the highest vesicle concentration tested (Figure S7b), the differences between different vesicle types became smaller as overall uptake by cells increased significantly. At this vesicle concentration the **1-DMPC+ β 4Gal-T1+TcTS** mixture (some sialic acid coating) had slightly higher values of internalisation when compared with the **1-DMPC+ β 4Gal-T1** mixture (LacNAc coating). However the non-functionalised vesicles (DMPC only) and **1-DMPC** (GlcNAc coating) had higher (at 3 h) or similar (24 h) levels of cellular uptake, which may suggest that high liposome concentrations produce much more extensive non-specific internalisation of vesicles by cells. However, this set of conditions needs to be studied in more detail to see if the observed differences are significant. These data suggest that vesicle concentrations need to be chosen careful in order to uncover any differences in cellular uptake of vesicle that have different cell-targeting coatings

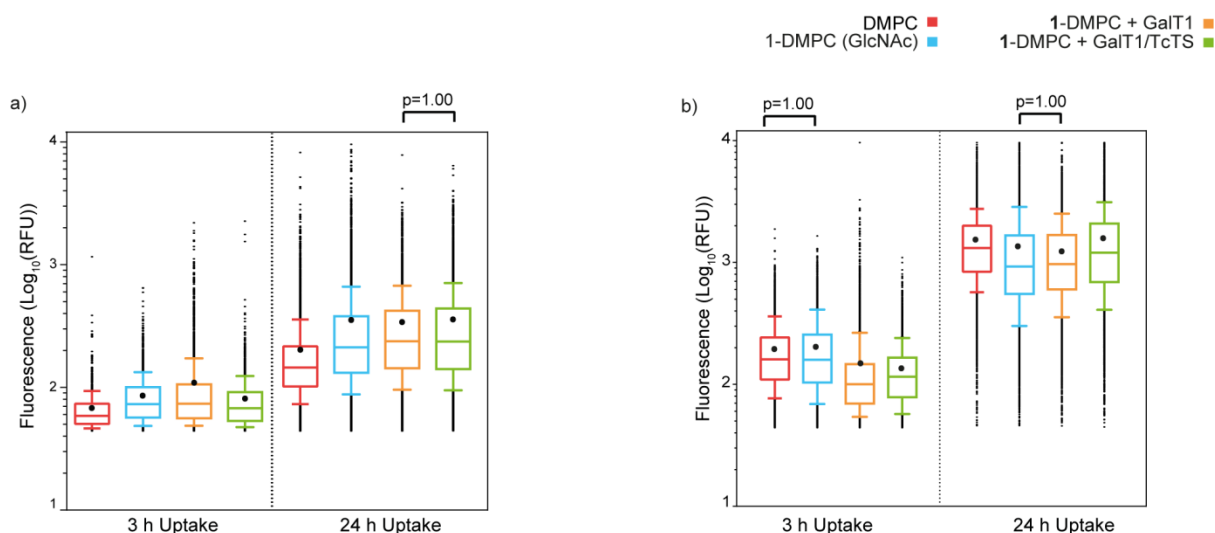


Figure S7. Quantification of cellular uptake of vesicles at a) 5.3 $\mu\text{g}/\text{mL}$ and b) 26.7 $\mu\text{g}/\text{mL}$ concentration. Samples were processed for flow cytometry and the fluorescence of each sample was analysed using a yellow-green laser for the excitation of the fluorophore rhodamine (vesicles). A total of 10,000 events were recorded for each sample. Flow cytometry results are presented as a box-plot chart (10th percentile, first quartile, median, third quartile and 90th percentile). The mean is represented as “•”. RFU: Relative fluorescence unit.

S10. Self-assembly of glycolipid **1** in MES buffer

The critical aggregation concentration of lipid has been measured as 15.4 nM.^[2] Given this value, it was anticipated the solubility of the lipid in MES buffer in the absence of added phospholipid would be low and would prevent the use of lipid **1** in suspension as a control, necessitating the use of **4** in solution as a control.

Nonetheless to assess the solubility of **1** and to determine what structures might be formed in buffer, lipid **1** (0.22 mg, 0.20 μmol) was dissolved in spectroscopic grade CHCl_3 (1 mL) and the solvent removed *in vacuo* to generate a thin film. The lipid film was further dried under reduced pressure to remove trace solvent, then rehydrated with MES buffer (1 mL, 50 mM, titrated with NaOH to give pH 7 at 22 $^{\circ}\text{C}$). This mixture was then sonicated at room temperature for 20 minutes, which gave a turbid suspension of lipid **1** (0.2 mM, the concentration used for the “one-pot” enzymatic transformation) (Figure S8).

This suspension was diluted 100-fold and 1000-fold (Figure S9) and the size of the particle measured using dynamic light scattering (DLS). These DLS measurement showed that the size of the particles in suspension ranged from 200 to 400 nm in diameter, a size that is too large for micellar aggregates and instead suggests that vesicles, tapes, sheet or undefined aggregates have been formed. This insolubility/self-assembly of lipid **1** in MES buffer alone prevented the measurement of the rate of transformation of the lipid **1** on its own and necessitated the use of the soluble analogue **4**.

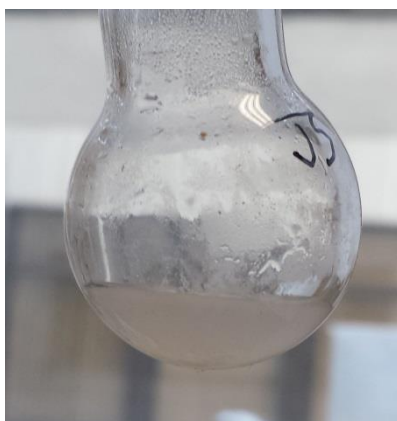


Figure S8: Photograph of a 0.2 mM suspension of lipid 1 in MES buffer (pH 7) at 22 °C.

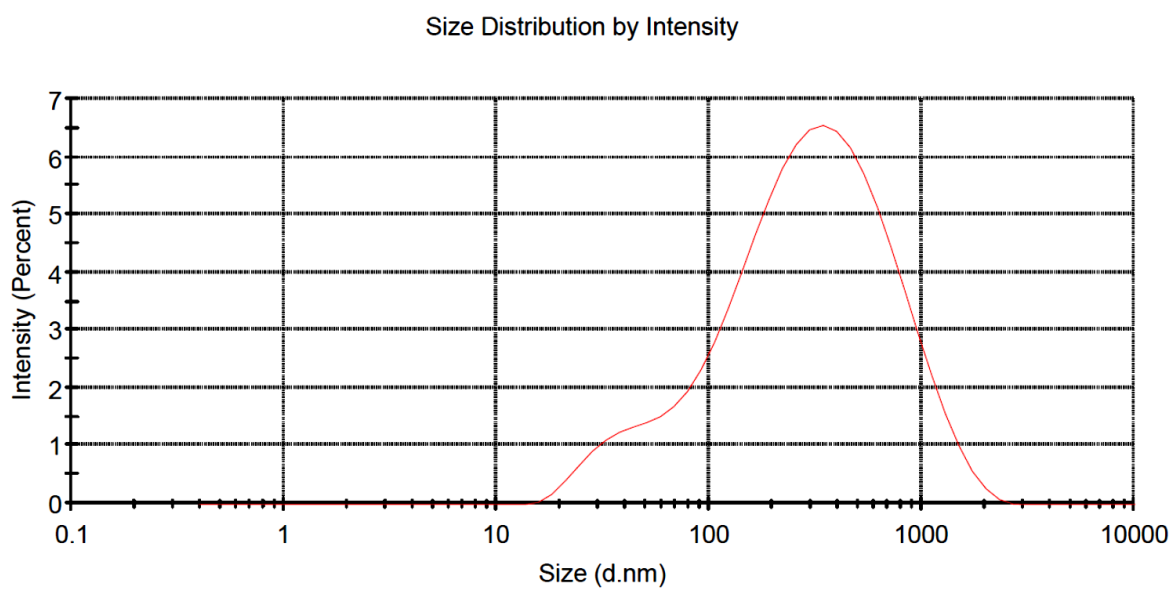


Figure S9: Dynamic light scattering data for a 0.2 μ M suspension of lipid 1 in MES buffer (pH 7) at 25 °C. Z-Average (d / nm) = 186.0; Pdl 0.476. Peak 1, size 391.3 nm, std. dev. 3.363 nm.

S11 References

- [1]. R. Šardzík, A. P. Green, N. Laurent, P. Both, C. Fontana, J. Voglmeir, M. J. Weissenborn, R. Haddoub, P. Grassi, S. M. Haslam, G. Widmalm and S. L. Flitsch, *J. Am. Chem. Soc.*, 2012, **134**, 4521–4524.
- [2]. G. T. Noble, F. L. Craven, J. Voglmeir, R. Šardzík, S. L. Flitsch and S. J. Webb, *J. Am. Chem. Soc.* 2012, **134**, 13010–13017.
- [3]. M. H. Wilbrink, G. A. ten Kate, S. S. van Leeuwen, P. Sanders, E. Sallomons, J. A. Hage, L. Dijkhuizen and J. P. Kamerling, *Appl. Environ. Microbiol.* 2014, **80**, 5984–5991.
- [4]. G. T. Noble, *PhD thesis*, University of Manchester, **2012**, pp 128.