

Cite this: *Chem. Commun.*, 2011, **47**, 2853–2855

www.rsc.org/chemcomm

COMMUNICATION

Stabilization of vesicular and supported membranes by glycolipid oxime polymers†

Mingming Ma, Soumitra Chatterjee, Meng Zhang and Dennis Bong*

Received 23rd November 2010, Accepted 5th January 2011

DOI: 10.1039/c0cc05137c

We report herein new synthetic glycolipid dimers and polymers that provide unprecedented stability to both supported (SLBs) and vesicular lipid bilayers against dehydration and serum exposure. These novel physical properties will enable pharmaceutical delivery and development of SLB bioanalytical devices.

The most widely used method to create long-circulating vesicle carriers¹ is membrane incorporation of lipid-anchored polyethylene glycol (PEG),² which sterically blocks serum protein binding, thus preventing lipid extraction, opsonization and immunoclearance. Similar protective effects were observed with GM1,³ though difficulties in accessing GM1 rendered its application in delivery impractical. To date, all long-circulating vesicle applications use PEG–lipids, with few improvements to the original system.¹ We report herein new, synthetically accessible glycolipid polymers that stabilize membranes to exposure to serum, air/freeze-drying and rehydration conditions. We anticipate that glycolipid systems of this type will be useful for the stabilization of synthetic membranes for delivery and SLB bioanalytical devices.⁴

We have used the α,α -disaccharide of glucose, trehalose, as a lipid crosslinker: tris-aminoxyether headgroup lipids were condensed with a synthetic diketo-trehalose derivative to yield oxime-linked glycolipid polymers (Fig. 1). α,α -Trehalose, which has previously been utilized in gene delivery,⁵ is an ideal disaccharide linker from considerations of chemical convenience and commercial accessibility. The non-reducing 1,1-glycoside skeleton is unreactive with aminoxyether lipids and permits symmetric regioselective installation of keto functionality at the C₆ and C_{6'} positions without the use of protecting groups. Transformation of the C₆ and C_{6'} primary alcohols into iodides⁶ was followed by a high-yielding 3-step sequence of thiolacetate addition, hydrolysis and conjugate addition to methylvinyl ketone (ESI†). Mono and tris-aminoxyether lipids were prepared by acylation of cholesterol or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-ethanolamine (POPE) with Boc-protected aminoxyether headgroups

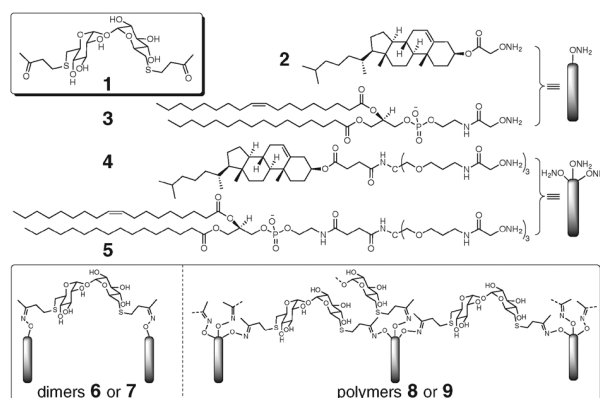


Fig. 1 Synthetic compounds used in this study: diketo-trehalose (inset **1**), monoaminoxyether lipids **2** and **3**, tris-aminoxyether lipids **4** and **5**. Oxime dimers **6** and **7** were derived from coupling of **1** with cholesterol **2** and phospholipid **3**, respectively, while oxime polymers were similarly derived from coupling of **1** with cholesterol **4** and phospholipid **5**, respectively.

(Fig. 1). Boc-cleavage with trifluoroacetic acid yielded the aminoxyether lipid TFA salts, which were directly reacted with diketo-trehalose **1** in either water/methanol/chloroform mixtures or acidic aqueous buffer to cleanly produce oxime dimers from the monoamino-oxoether lipids, and branched sugar–lipid oxime polymers from the tris-aminoxyether lipids.

While *in situ* reaction of pre-formed vesicles containing aminoxyether lipids resulted in large vesicle aggregates, hydration of mixed lipid films containing defined mole fractions of oxime-linked glycolipids yielded well-behaved suspensions. Both oxime dimers and polymers were found to be stable at 37 °C at neutral pH (PBS) for several days, as monitored by mass spectroscopy.^{6b} Polymer size, tunable by reaction time and concentration, was determined by diffusion ordered NMR (DOSY) using PMMA molecular weight standards for calibration (Fig. 2A).⁷ Larger (20 kD) cholesterol polymers were less soluble than the 7 kD cholesterol oligomer (~hexamer) (**8**), while POPE-derived 20 kD polymer (~15mer) (**9**) and both sterol and POPE dimers (**6** and **7**, respectively) were soluble in methanol/chloroform mixtures. Organic solvent solubility permitted the preparation of mixed lipid films, which could be hydrated into vesicles. Notably, hydration and extrusion of a mixed lipid film containing ePC and either

Department of Chemistry, The Ohio State University,
100 W. 18th Avenue, Columbus, OH 43210, USA.
E-mail: bong@chem.osu.edu

† Electronic supplementary information (ESI) available: Synthetic and experimental procedures, compound characterization, DOSY spectra, DLS and zeta potential data. See DOI: 10.1039/c0cc05137c

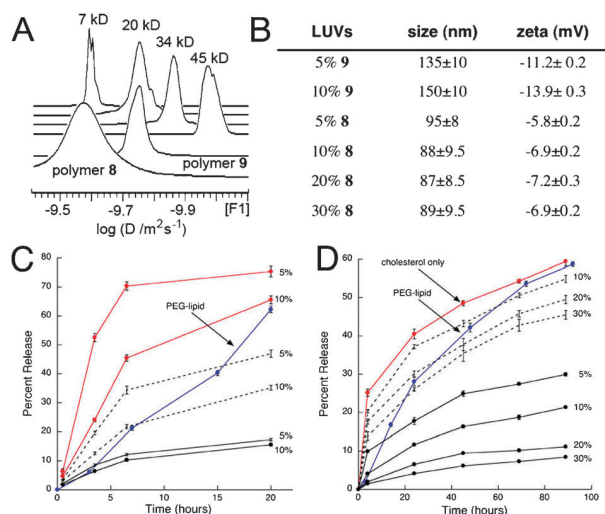


Fig. 2 (A) Diffusion coefficients for PMMA standards and oxime polymers derived from cholesterol (**8**) and phospholipid (**9**). (B) DLS and zeta potential of ePC LUVs with **8** or **9**. Calcein release on exposure to 30% FBS at 37 °C from (C) ePC LUVs with the indicated mole percent POPG (●), 5% PEG-DSPE (●), dimer **7** (---) and 20 kD PE-polymer **9** (—); (D) ePC LUVs with 30% cholesterol (●), 30% cholesterol, 5% PEG-DSPE (●), cholesterol dimer **6** (---) and cholesterol polymer **8** (—). Total cholesterol (free and glycolipid) was constant at 30%. Measurement made over 20 hours and 100 hours in (C) and (D), respectively.

glycolipid polymer required elevated temperatures (50 °C) while ePC films are easily handled at room temperature. Neither polymer could be suspended in water in the absence of a lipid host environment. While this is unsurprising for the sterol polymer, we expected the phospholipid glycopolymer **9** to be more soluble. These altered properties may derive from the rigidity of the trehalose linker which may inhibit proper hydrophobic burial and suspension in water. Notably, the phospholipid dimer **7** could easily be hydrated to form a suspension, underscoring the differences in dimer and polymer of both physical properties and function.

Szoka and co-workers have shown that membranes with multiply-anchored cholesterol lipids exhibit enhanced stability.⁸ Consistent with these findings, cholesterol glycopolymer **8** in LUVs provided the most effective stabilization in 30% fetal bovine serum at 37 °C as judged by a contents release assay⁹ (Fig. 2). The cholesterol and phospholipid anchored polymers **8** and **9** both yielded significant protection in FBS relative to controls, as did the respective trehalose–lipid dimers **6** and **7**. The superior stabilization exhibited by the cholesterol-based glycopolymer likely derives from the greater inherent stability of cholesterol-containing membranes.¹⁰ The phospholipid series was compared to control LUVs wherein negatively charged phospholipid (POPG) replaced 20 kD polymer **9**, which has one negative charge per lipid anchor; indeed, dimer **7** and polymer **9**–LUVs had zeta potentials similar to LUVs containing the same mole fraction of POPG (Fig. 2B). Likewise, cholesterol dimer **6** and polymer **8**–LUVs had sizes and low magnitude zeta potentials similar to LUVs composed of only zwitterionic PC and neutral cholesterol lipids. A fluid phase membrane composed of 70% egg PC (ePC) and 30 mole

percent sterol (cholesterol/**8**) was used to allow release to be detected on the time scale of a few days. Vesicle stabilization by the glycolipid derivatives was strongly affected by degree of oligomerization and solubility. With both cholesterol and phospholipid anchors, the trehalose lipid dimers offered significant stabilization in FBS, but the polymeric trehalose–lipids always yielded superior protection per mole lipid anchor. Though there is clearly an advantage to structures larger than a dimer, larger oligomer or polymers are limited by their ability to incorporate into the lipid membrane. Thus, it was difficult to achieve mole percent incorporation levels higher than 10% for **9** and 30% for **8**.

A unique property of 30% **8** in DPPC or hydrogenated ePC LUVs is that they may be lyophilized and rehydrated with minimal contents release (average of three trials was ~10% contents loss) when calcein dye was encapsulated; further, no significant changes in vesicular size or structure were observed by DLS and cryo-TEM (Fig. 3). However, total contents loss and extensive fusion was observed when cholesterol or dimer **6** was used in place of **8**, or if hydration was attempted at elevated temperatures (~70 °C). Sugar solutions (10 : 1 sugar : lipid) are known to protect LUVs from contents loss and fusion upon lyophilization and rehydration, and we find that covalent presentation of polymeric trehalose on multiple lipid anchors confers significant membrane anhydrobiotic and cryo-protection at sugar : lipid ratios of 0.1–0.3. Rehydration of freeze-dried gel phase LUVs without size change requires steric inhibition of vesicle fusion, which appears to be provided by the membrane-anchored trehalose–cholesterol oligomers.¹¹ Cholesterol–polymer containing vesicles deposited poorly on glass, resulting in low yields of SLB formation, while LUVs containing PE-derived **7** and **9** readily deposited, possibly by virtue of higher membrane fluidity. Though dimer **7** preserved SLBs from delamination (uncoating from the glass

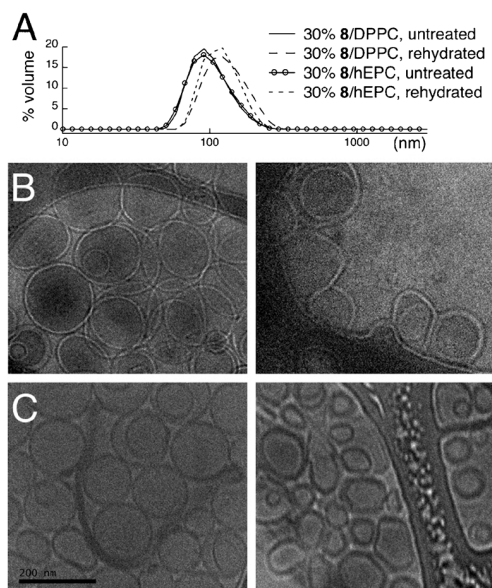


Fig. 3 LUVs containing 30% **8** analyzed using (A) dynamic light scattering (173°) and cryo-TEM (B, C). Cryo-TEM images of (left) freshly hydrated and (right) lyophilized and resuspended: (B) DPPC LUVs and (C) hEPC (scale bar = 200 nm).

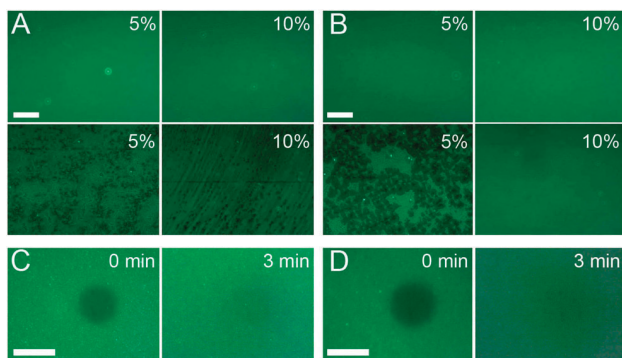


Fig. 4 Supported lipid bilayers containing (A) **7** and (B) **9**, mole percent as indicated. Top rows (A) and (B): freshly formed SLBs; bottom rows: after drying and rehydration, 10× magnification, scale bar = 100 μm. FRAP in SLBs with 10% **9**, before dehydration (C) and after dehydration/rehydration (D), 40× magnification, scale bar = 40 μm.

surface) during dehydration–rehydration, considerable surface scarring occurred, whereas PE–polymer **9** preserved both surface uniformity and membrane fluidity, as judged by fluorescence recovery after photobleaching (FRAP) at just 10 mole percent loading of **9** (Fig. 4), similar to prior reports of protection of lipid monolayers by synthetic trehalose lipids.¹² As with vesicular membranes, polymeric glycolipid **9** offered superior anhydrobiotic SLB protection relative to dimer **7**, again suggesting that presentation of covalently clustered sugars as afforded by the polymeric structure of **9** is key. Stabilization of membranes to physical insults over a wide range of hydration is hugely enabling for the development of bilayer-based devices.¹³ The current method provides a strategy that may be suitable for stabilization of free-standing membranes. Trehalose exhibits exceptional function among disaccharides with regard to protection against anhydrobiotic and cryogenic of SLBs,¹⁴ protein¹⁵ and vesicles,¹¹ leading to its use in pharmaceutical formulations;^{16,17} synthetic and native trehalose lipids are known similarly to protect SLBs.^{12,18} Trehalose also possesses a number of unique physical properties: it has the largest hydrated radius of any disaccharide, the highest glass transition temperature, reversible, hydration-dependent polymorphism, and kosmotropic function in water. These physical properties are thought to allow trehalose to protect biomolecular assemblies (such as membranes) in a glassy solid sugar coating that is stable to varying hydration levels.¹⁵ We are continuing to investigate the significance of sugar structure to membrane stability, though we note that the present trehalose-based system is conveniently accessible and exhibits unprecedented stability over a wide range of hydration. Our glycolipid strategy complements existing stabilization approaches^{1,19} and allows access to neutral or charged glycolipid polymer membranes that have unique anhydrobiotic and cryogenic stability. PEG-protected vesicles are the only marketed immunoevasive liposomal drug carriers but cannot be freeze-dried without external protectant,^{1,20}

we are optimistic that glycolipid polymer systems may be further engineered to completely block contents loss while maintaining vesicular shape and size during freeze-drying. Furthermore, though some glycosylated lipids are highly immunogenic,²¹ mycobacteria-derived trehalose dimycolate lipid (TDM) is itself nonimmunogenic when membrane anchored, suggesting that synthetic trehalose lipids may be similarly inert.²² We anticipate that the glycolipid polymer stabilized membranes reported herein will be of use as long-circulating drug carriers. Overall, these data indicate that glycolipid polymers stabilize vesicular and supported lipid membranes to anhydrobiotic and cryogenic conditions, which has not been previously demonstrated with lipid polymerization²³ or any other strategy. Thus, membrane-protection with glycolipids and related biomaterials holds promise as an enabling technology for delivery and membrane-based devices.²⁴

Notes and references

- 1 T. Kaasgaard and T. L. Andresen, *Expert Opin. Drug Delivery*, 2010, **7**, 225–243; M. L. Immordino, F. Dosio and L. Cattel, *Int. J. Nanomed.*, 2006, **1**, 297–315.
- 2 T. M. Allen, C. Hansen, F. Martin, C. Redemann and A. Yau-Young, *Biochim. Biophys. Acta, Biomembr.*, 1991, **1066**, 29–36.
- 3 T. M. Allen and A. Chonn, *FEBS Lett.*, 1987, **223**, 42–46.
- 4 E. Reimhult and K. Kumar, *Trends Biotechnol.*, 2008, **26**, 82–89.
- 5 S. Srinivasachari, Y. Liu, G. Zhang, L. Prevet and T. M. Reineke, *J. Am. Chem. Soc.*, 2006, **128**, 8176–8184.
- 6 (a) J. M. G. Fernandez, C. O. Mellet, J. L. J. Blanco, J. F. Mota, A. Gadelle, A. Coste-Sarguet and J. Defaye, *Carbohydr. Res.*, 1995, **268**, 57–71; (b) J. Kalia and R. T. Raines, *Angew. Chem., Int. Ed.*, 2008, **47**, 7523–7526.
- 7 A. Chen, D. Wu and C. S. Johnson, *J. Am. Chem. Soc.*, 1995, **117**, 7965–7970.
- 8 Z. Huang and F. C. Szoka, Jr., *J. Am. Chem. Soc.*, 2008, **130**, 15702–15712.
- 9 R. R. C. New, *Liposomes: A Practical Approach*, Oxford, NY, 1990.
- 10 R. B. Gennis, *Biomembranes*, 1990.
- 11 J. H. Crowe, L. M. Crowe, A. E. Oliver, N. Tsvetkova, W. Wolkers and F. Tablin, *Cryobiology*, 2001, **43**, 89–105.
- 12 C. W. Harland, Z. Botyanszki, D. Rabuka, C. R. Bertozzi and R. Parthasarathy, *Langmuir*, 2009, **25**, 5193–5198.
- 13 Y. Deng, Y. Wang, B. Holtz, J. Li, N. Traaseth, G. Veglia, B. J. Stottrup, R. Elde, D. Pei, A. Guo and X. Y. Zhu, *J. Am. Chem. Soc.*, 2008, **130**, 6267–6271.
- 14 F. Albertorio, V. A. Chapa, X. Chen, A. J. Diaz and P. S. Cremer, *J. Am. Chem. Soc.*, 2007, **129**, 10567–10574.
- 15 N. K. Jain and I. Roy, *Protein Sci.*, 2009, **18**, 24–36.
- 16 United States Pat., 6221385, 1997.
- 17 J. F. Willart and M. Descamps, *Mol. Pharmacol.*, 2008, **5**, 905–920.
- 18 C. W. Harland, D. Rabuka, C. R. Bertozzi and R. Parthasarathy, *Biophys. J.*, 2008, **94**, 4718–4724.
- 19 S.-M. Lee, H. Chen, C. M. Dettmer, T. V. O'Halloran and S. T. Nguyen, *J. Am. Chem. Soc.*, 2007, **129**, 15096–15097.
- 20 G. L. Plosker, *Drugs*, 2008, **68**, 2535–2551.
- 21 G. De Libero and L. Mori, *Prog. Lipid Res.*, 2010, **49**, 120–127.
- 22 R. L. Hunter, M. R. Olsen, C. Jagannath and J. K. Actor, *Ann. Clin. Lab Sci.*, 2006, **36**, 371–386.
- 23 A. Mueller, B. Bondurant and D. F. O'Brien, *Macromolecules*, 2000, **33**, 4799–4804.
- 24 F. Albertorio, A. J. Diaz, T. Yang, V. A. Chapa, S. Kataoka, E. T. Castellana and P. S. Cremer, *Langmuir*, 2005, **21**, 7476–7482.