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Development and characterization of anionic liposaccharides for enhanced oral drug delivery

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ABSTRACT

The aim of this study was to synthesize charged amphoteric molecules, which after complexation with poorly bioavailable drugs would have the potential to improve their oral uptake. Novel anionic liposaccharide derivatives containing D-glucose and lipoamino acids were synthesized by solution phase peptide synthesis. High sensitivity isothermal titration microcalorimetry was used to determine the critical aggregation concentration and the thermodynamic profiles. Hemolytic and cytotoxic activities of the liposaccharides were studied and they revealed that the liposaccharides were non-toxic at the concentration used for oral administration. Mixing a model drug, tobramycin, with the liposaccharide containing two lipids formed aggregates around 200 nm, which increased tobramycin partitioning between *n*-octanol/water. The results suggested that the studied liposaccharide with two lipids was safe to apply biologically and may have an absorption enhancing activity on hydrophilic, orally poorly available drugs.

1. Introduction

The most convenient and preferred drug administration is the oral route due to patient convenience, compliance, ease of administration and low cost. However, the administration of many drugs (e.g. tobramycin, piperacillin) has been limited to the parenteral route. This is mainly due to the low bioavailability caused by poor solubility in the intestinal tract and/or lack of permeation through the epithelial cells (Hosny et al., 2002). Many approaches have been investigated to overcome these problems, for example the co-administration of various absorption enhancers, like surfactants, bile salts, calcium chelating agents, fatty acids, cyclodextrines and chitosans (Simerska et al., 2011). The mechanisms of absorption enhancers were studied previously using human epithelial colorectal adenocarcinoma cells (Caco-2 cell) (Lindmark et al., 1995). The use of these absorption enhancers in oral formulations facilitates permeation through intestinal membranes and thus enables the attainment of the required therapeutic levels. The ease of incorporation of a safe and effective absorption enhancer into the conventional oral dosage form, thereby circumventing the need for the sophisticated and costlier drug delivery systems of parenteral therapy, has made absorption enhancers commercially viable. It was previously shown by our group that the co-administration of the hydrophilic drug gentamicin and liposaccharide-based absorption enhancers improved absorption of the parent drug in rat intestine (Ross et al., 2004).

In the present study, novel anionic liposaccharide derivatives were designed and synthesized to comprise one or two lipoamino acids (LAAs) and a D-glucose derivative. LAAs are synthetic amino acids with a lipophilic alkyl side chain (Wong and Toth, 2001). The advantage of incorporation of these lipid moieties into the absorption enhancing structures is their capacity to modulate the physico-chemical properties of the system. This can be achieved by their ability to easily control the lipophilicity (e.g. number of LAA incorporated, length of the alkyl side chain of the LAA) of the formed complex which is directly correlated to the oral absorption of the drug-enhancer system (Drouillat et al., 1998). It was recently confirmed that LAAs form amphiphilic ion pairs with the macrolide class antibiotic, erythromycin, with no alteration to its antibacterial activity (Pignatello et al., 2011). Utilization of LAAs has also been proven to enhance the oral absorption of a wide range of therapeutic antibiotics (e.g. piperacillin (Abdelrahim et al., 2009; Violette et al., 2008; Falconer and Toth, 2007) and gentamicin (Ross et al., 2004)). Highly lipophilic compounds on the other hand are poorly water soluble. To balance the level of lipophilicity and to utilize active or facilitated glucose transport systems during absorption, these lipid moieties were glycosylated. Since both carbohydrate and LAAs are biocompatible and biodegradable, our liposaccharide-based system offers significant benefits in terms of regulatory approval. Another component of the liposaccharide derivatives is a carboxylic group converted to a sodium salt, thus promoting surfactant and ion-pairing characteristics and

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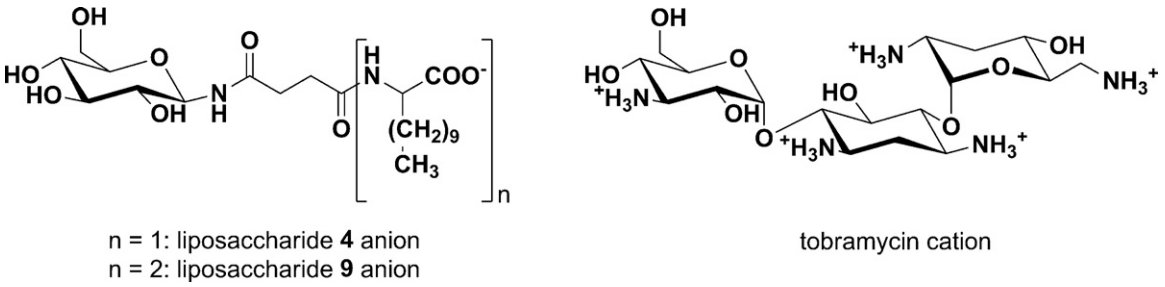


Fig. 1. Charged liposaccharides **4** and **9** associated with the model drug tobramycin.

enhancing passive diffusion of a drug-absorption enhancer complex. In addition, the presence of a sodium salt modulates the solubility of the liposaccharide derivatives during oral administration (Serajuddin, 2007; Derry et al., 2009).

The potential interactions of the anionic liposaccharides with erythrocyte membranes were investigated in this study using hemolytic experiments. These experiments are commonly used as a model for membrane interactions as the erythrocytes are readily available and their lysis can be easily measured by spectroscopy (Pignatello et al., 2007). The hemolytic values may be overestimated when measured in vitro due to many factors (e.g. the lack of mucus reducing the access of the enhancer to the epithelium and/or its complexation with intestinal lining fluid components and/or rapid clearance mechanisms that occur in vivo). Although the intestinal epithelium may be less sensitive in vivo, the in vitro hemolysis investigation helps to identify the liposaccharides, which are less toxic and therefore safer and more biocompatible for use in oral drug delivery.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess the cell viability by measurement of the mitochondrial dehydrogenase activity in a colorimetric assay. MTT is a tetrazolium salt, which is cleaved by mitochondrial dehydrogenases to produce a dark blue product when measured spectrophotometrically. Damaged or dead cells show reduced or no dehydrogenase activity.

Tobramycin, an aminoglycoside antibiotic used mainly to treat Gram-negative bacterial infections, is a highly polar cationic drug with poor oral bioavailability (Hombach et al., 2008). Tobramycin or its sulphate is not taken up in the gastrointestinal tract and is not available for oral administration. The partitioning of tobramycin in *n*-octanol/water was studied in two different molar ratios (1:1 and 1:7) of tobramycin/liposaccharide (Fig. 1).

2. Materials and methods

Trifluoroacetic acid (TFA) and diisopropylethyl amine (DIPEA), were purchased from Auspep (Melbourne, VIC, Australia). *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetra-methyl-uronium-hexafluoro-phosphate (HBTU) and di-*tert*-butyldicarbonate (Boc_2O) were obtained from GL Biochem Ltd. (Shanghai, China). N^{α}-Boc-protected amino acids were supplied by Novabiochem (Laufelfingen, Switzerland). Palladium (10 wt% on carbon) was purchased from Lancaster Synthesis (Lancashire, England), and Amberlite ion exchange resin (IR-120) [H^-] was provided by British Drug Houses (BDH) Ltd. (England). Ultrapure gases (N_2, H_2, Ar) were supplied by BOC Gases (Brisbane, QLD, Australia). Silica for flash chromatography (silica gel 60, 230–400 mesh) was obtained from Lomb Scientific (Taren Point, NSW, Australia). Deuterated solvents (d_1-DCl_3 and DMSO-d_6) were manufactured by Cambridge Isotope Laboratories Inc. (Andover, MA, USA). All other reagents were purchased in analytical grade or higher purity from Sigma–Aldrich (Castle Hill, NSW, Australia) or Merck Pty. Ltd. (Kilsyth, VIC, Australia). Solvents were freshly distilled and dried prior to use and all moisture-sensitive reactions were carried out under inert atmosphere (N_2/Ar) using oven-dried glassware. Reactions were carried out at a room temperature unless otherwise specified. Thin-layer chromatography (TLC) was performed on silica gel 60 F254 aluminium sheets (Merck, Darmstadt, Germany), and compounds were visualized by either ninhydrin dip (0.1% ninhydrin in ethanol) or cerric sulfate dip (15% aqueous H_2SO_4 saturated with cerric sulfate). All TLC plates were developed by heating after treatment with the developing agent. Melting points were measured with a capillary apparatus and are uncorrected.

Nuclear magnetic resonance (NMR) spectra were recorded at room temperature in deuterated chloroform ($CDCl_3$) solutions (unless otherwise indicated). ^1H and ^{13}C NMR spectra were recorded using a Bruker AM 500 instrument operating at a field of 500 MHz. Chemical shifts are reported in parts per million (ppm) downfield from internal tetramethylsilane (TMS). Signal multiplicities are represented as singlet (s), doublet (d), double doublet (dd), triplet (t), quartet (q), quintet (quint), multiplet (m), broad (br), and broad singlet (brs). Liquid chromatography mass spectroscopy (LC–MS/MS) data were measured on a Waters 2790 instrument using positive mode electrospray ionization. Analytical results were within ±0.4% of the theoretical values for the formula given unless otherwise indicated. The mobile phase for mass spectrometry and HPLC was a mixture of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in 90% acetonitrile and 10% water). Mass spectra were recorded on a PerkinElmer Sciex API 3000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada) operating in positive ion electrospray mode (ESI-MS). High resolution mass spectrometry (HRMS) data were obtained on a QSTAR Pulsar, a high performance, hybrid quadrupole Time-of-Flight mass spectrometer (Applied Biosystems) operating in positive ion electrospray mode.

2.1. Synthesis

2.1.1. Methyl-(2-(N-(4-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoate (**3**)

N-(4-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylamino) succinic acid **1** (Kellam et al., 1998) (1.00 g, 2.23 mmol), HBTU (0.50 g, 2.68 mmol) and DIPEA (0.77 ml, 4.47 mmol) were dissolved in dry dichloromethane (DCM) (50 ml), LAA **2** (Bai et al., 2001) (0.51 g, 2.23 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. Then it was washed with 5% hydrochloric acid (HCl) (2× 50 ml), a saturated solution of sodium bicarbonate ($NaHCO_3$) (2× 50 ml), dried over magnesium sulfate ($MgSO_4$), and filtered. The residual solvent was evaporated under vacuum and the crude product **3** was purified by column chromatography.

Methyl-(2-(*N*-(4-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoate **3** (1.03 g, 1.57 mmol) was synthesized in 70% yield. R_f = 0.6 in methanol: DCM,

1:9 (v/v); 1:1 mixture of diastereoisomers; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.14–7.04 (1H, m, amide NH), 6.98–6.94 (1H, m, amide NH), 5.26–5.23 (1H, m, H-1 (glucose)), 5.19–5.16 (1H, m, H-2 (glucose)), 5.03–4.99 (1H, m, H-3 (glucose)), 44.89–4.87 (1H-m, H-5 (glucose)), 4.44–4.42 (1H, m, H-4 (glucose)), 4.85–3.82 (1H, t, J = 4.1 Hz, CH (lipid)), 3.75–3.74 (2H, m, H-6a,b (glucose)), 3.69 (3H, s, OCH_3) 2.69–2.59 (4H, m, $2CH_2$ (succinic)), 2.09, 2.05, 2.02,1.95, (12H, 4s, $4CH_3CO$ (glucose)), 1.80–1.68 (2H, m, β-CH_2 (lipid)), 1.30–1.25 (16H, m, $8CH_2$ (lipid)), 0.89–0.86 (3H, t, J = 6.25 Hz,CH_3 (lipid)); ^{13}C NMR (500 MHz, $CDCL_3$) δ; 173.81, 173.78, 173.62, 173.24, 173.20, 173.13, 172.63, 172.63, 172.59, 172.27, 171.19, 171.16, 171.12, 171.09, 170.97, 170.94, 170.13, 169.77, 165.81, 159.34, 159.02, 158.69, 158.37, 118.18, 115.91, 113.63, 111.36, 78.12, 73.41, 72.71, 72.65, 70.42, 67.96, 61.96, 61.65, 52.98, 52.87, 52.73, 52.67, 38.62, 31.80, 31.72, 31.64, 31.47, 31.23, 31.17, 30.54, 29.37, 29.34, 29.17, 29.12, 28.92, 25.20, 25.10, 22.49, 20.45, 20.43, 20.34, 20.26, 20.24, 13.89, 13.98; HRMS calculated for [$C_{31}H_{50}N_2NaO_{13}$] [M+Na] 681.3211, found 681.3211.

2.1.2. (2-(*N*-(4-(β-D-Glucopyranosylamino)succinyl))amino-D,L-dodecanoic acid (**4a**)

Liposaccharide **3** (1.30 g, 1.97 mmol) was dissolved in methanol (30 ml) and the pH was adjusted to 12 using 1 M sodium methoxide ($NaOCH_3$) and stirred for 2 h; then water (10 ml) was added and the pH was readjusted to 13. The solution was stirred at room temperature for additional 12 h. Upon the completion (checked by TLC), the reaction mixture was acidified using Amberlite resin IR-120 [H^+] until an acidic pH was obtained. The reaction mixture was filtered and the filtrate was evaporated under vacuum. The residue was lyophilized from acetonitrile:water (1:1) to give a de-methylated liposaccharide **4a**.

(2-(*N*-(4-(β-D-Glucopyranosylamino)succinyl))amino-D,L-dodecanoic acid **4a** (0.84 g, 1.76 mmol) was synthesized in 89% yield. mp 90 °C; 1:1 mixture of diastereomers; ^{1}H NMR (500 MHz, MeOD) δ 4.23–4.20 (1H, m, CH (lipid)), 3.73–3.71 (1H, m, H-2 (glucose)), 3.60–3.56 (1H, m, H-3 (glucose)), 3.55–3.52 (2H, m, H-4 and H-5 (glucose)), 3.32–3.21 (4H, m, 4OH (glucose)), 3.20–3.14 (2H, m, H-$6_{a,b}$(glucose)), 2.52–2.35 (4H, m, $2CH_2$ (succinic)), 1.75–1.72 (2H, m, β-CH_2 (lipid)), 1.37–1.27 (16H, m, $8CH_2$ (lipid)), 0.80 (3H, t, J = 6.9 Hz, CH_3 (lipid)); ^{13}C NMR (500 MHz, MeOD) δ 175.56, 174.77, 174.66, 174.49, 81.07, 79.57, 78.85, 78.84, 74.00, 71.37, 62.68, 53.88, 53.81, 53.78, 53.74, 52.64, 33.02, 32.52, 32.50, 32.10, 31.85, 31.85, 31.39, 31.07, 30.66, 30.66, 30.63, 30.60, 30.46, 30.41, 30.26, 30.19, 30.13, 30.08, 26.80, 23.69, 14.42; HRMS calculated for [$C_{22}H_{39}N_2O_9$] [M+H], 475.2656; found 475.2661.

2.1.3. Sodium (2-(N-(4-(β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoate (**4**)

The free acid **4a** (0.47 g, 1.00 mmol) was suspended in water (50 ml), $NaHCO_3$ (0.08 g, 1.00 mmol) was added and the reaction mixture was sonicated. After lyophilization, the compound **4** was obtained in quantitative yield as a white powder.

Sodium (2-(*N*-(4-(β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoate **4** (0.49 g, 0.98 mmol) mp 120 °C; 1:1 mixture of diastereomers; ^{1}H NMR (500 MHz, MeOD) δ 4.23–4.20 (1H, m, CH (lipid)), 3.73–3.71 (1H, m, H-2 (glucose)), 3.60–3.56 (1H, m, H-3 (glucose)), 3.55–3.52 (2H, m, H-4 and H-5 (glucose)), 3.32–3.21 (4H, m, 4OH (glucose)), 3.20–3.14 (2H, m, H-$6_{a,b}$(glucose)), 2.52–2.35 (4H, m, $2CH_2$ (succinic)), 1.75–1.72 (2H, m, β-CH_2 (lipid)), 1.37–1.27 (16H, m, $8CH_2$ (lipid)), 0.80 (3H, t, J = 6.9 Hz, CH_3 (lipid)); ^{13}C NMR (500 MHz, MeOD) δ 175.56, 174.77, 174.66, 174.49, 81.07, 79.57, 78.85, 78.84, 74.00, 71.37, 62.68, 53.88, 53.81, 53.78, 53.74, 52.64, 33.02, 32.52, 32.50, 32.10, 31.85, 31.85, 31.39, 31.07, 30.66, 30.63, 30.60, 30.46, 30.41, 30.26, 30.19, 30.13, 30.08, 26.80, 23.69, 14.42; HRMS calculated for [$C_{22}H_{39}N_2O_9$] [M+Na], 475.2656; found 475.2661.

2.1.4. Methyl-2-(N-(2-(N-tert-butyloxycarbonyl)amino-D,L-dodecanoyl)amino-D,L-dodecanoate (**6**)

Boc-protected LAA **5** (1.00 g, 3.17 mmol), HBTU (0.72 g, 3.80 mmol) and DIPEA (1.09 ml, 6.27 mmol) were dissolved in dry DCM (50 ml). Methyl-protected LAA **2** (0.72 g, 3.17 mmol) was added and the mixture was stirred at room temperature for 12 h. The reaction mixture was washed successively with 5% HCl (2× 50 ml), a saturated solution of $NaHCO_3$ (2× 50 ml), dried over $MgSO_4$, and filtered. The residual solvent was evaporated under vacuum and the crude product was purified by column flash chromatography to give pure product **6** as a colorless oil.

Methyl-2-(*N*-(2-(*N*-*tert*-butyloxycarbonyl)amino-D,L-dodecanoyl)amino-D,L-dodecanoate **6** (di-LAA) (1.24 g, 2.36 mmol) R_f = 0.6 in ethyl acetate:hexane, 1:2 (v/v); 1:1 mixture of diastereomers; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.05–6.96 (1H, m, amide NH), 4.52 (1H, t, J = 9.70, CH (lipid)), 4.11 (1H, t, J = 9.74, CH (lipid)), 3.66 (3H, s, OCH_3), 1.76–1.73 (2H, m, β-CH_2 (lipid)), 1.61–1.54 (2H, m, β-CH_2 (lipid)), 1.38 (9H, s, Boc), 1.30–1.25 (32H, m, 2($8CH_2$) (lipid)), 0.83 (6H, t, J = 6.85 Hz, $2CH_3$ (lipid)); ^{13}C NMR (500 MHz, $CDCL_3$) δ 172.70, 172.60, 172.09, 155.54, 155.48, 79.43, 54.23, 51.95, 51.89, 32.56, 32.44, 32.04, 31.70, 29.40, 29.39, 29.32, 29.22, 29.13, 29.12, 29.03, 29.01, 28.11, 25.34, 25.14, 22.47, 13.87; HRMS calculated for [$C_{30}H_{58}N_2NaO_5$] [M+Na] 549.4243, found 549.4238.

2.1.5. Methyl-2-(N-(2-amino-D,L-dodecanoyl)amino-D,L-dodecanoate (**7**)

Di-LAA **6** (1.50 g, 2.85 mmol) was dissolved in TFA:DCM (1:1) and stirred for 1 h. The reaction mixture was diluted with DCM (50 ml), evaporated and washed with a saturated solution of $NaHCO_3$ (2× 50 ml), dried over $MgSO_4$, and filtered. The residual solvent was evaporated under vacuum to produce di-LAA **7** as a colorless oil.

Methyl-2-(*N*-(2-amino-D,L-dodecanoyl)amino-D,L-dodecanoate **7** (1.15 g, 2.84 mmol) was obtained in a quantitative yield. 1:1 mixture of diastereomers; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.71–7.63 (1H, m, amide NH), 4.52 (1H, t, J = 9.70, CH (lipid)), 4.11 (1H, t, J = 8.97 CH (lipid)), 3.66 (3H, s, OCH_3), 3.33 (1H, s, NH_2), 1.79–1.75 (2H, m, β-CH_2 (lipid)), 1.62–1.57 (2H, m, β-CH_2 (lipid)), 1.30–1.25 (32H, m, 2($8CH_2$) (lipid)), 0.83 (6H, t, J = 6.85 Hz, $2CH_3$ (lipid)); ^{13}C NMR (500 MHz, $CDCL_3$) δ 174.85, 172.93, 172.86, 54.96, 51.90, 51.61, 51.49, 34.95, 32.23, 32.10, 31.71, 29.41, 29.38, 29.36, 29.34, 29.31, 29.28, 29.21, 29.13, 29.00, 28.99, 25.54, 25.47, 25.21, 25.13, 22.47, 13.88; HRMS calculated for [$C_{25}H_{51}N_2O_3$] [M+H] 427.3900, found 427.3894.

2.1.6. Methyl 2-(N-(2-(N-(4-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoyl)-amino-D,L-dodecanoate (**8**)

N-(4-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosylamino) succinic acid **1** (1.20 g, 2.68 mmol), HBTU (0.60 g, 3.22 mmol) and DIPEA (0.93 ml, 5.34 mmol) were dissolved in dry DCM (50 ml). Di-LAA **7** (1.14 g, 2.68 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was washed successively with 5% HCl (2× 50 ml), a saturated solution of $NaHCO_3$ (2× 50 ml), dried over $MgSO_4$, and filtered. The residual solvent was evaporated under vacuum and the crude product was purified by column chromatography to give a liposaccharide **8** as a colorless oil.

Methyl 2-(*N*-(2-(*N*-(4-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoyl)-amino-D,L-dodecanoate **8** (1.48 g, 1.73 mmol) was synthesized in 65% yield; R_f = 0.6 methanol:DCM, 1:9 (v/v); 1:1 mixture of diastereomers; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.20–7.18 (1H, m, amide NH), 7.05–7.03 (1H, m, amide NH), 5.24–5.20 (2H, m, H-1 and H-2 (glucose)), 4.99 (1H, t, J = 9.75, CH (lipid)), 4.88 (1H, t, J = 9.45, CH (lipid)), 4.47–4.40 (2H, m, H-3 and H-4 (lipid)), 4.23–4.21 (1H, m, H-5 (glucose)), 4.02–3.99 (1H, m, H-6_a (glucose)), 3.77–3.75 (1H, m, H-6_b (glucose)), 3.64 (3H, s, OCH_3), 2.50–2.46 (4H, m, $2CH_2$ (succinic)), 1.99, 1.95, 1.94, 1.93 (12H, 4s, $4CH_3CO$ (glucose)), 1.74–1.72 (2H, m, β-CH_2 (lipid)), 1.60–1.53 (2H, m, β-CH_2 (glucose)), 1.201.17 (32H, m, 2($8CH_2$) (lipid)), 0.83 (6H, t, J = 6.25 Hz, $2CH_3$ (lipid)); ^{13}C NMR (500 MHz, $CDCL_3$) δ 172.74, 172.70, 172.56, 172.52, 171.68, 171.62, 170.55, 170.49, 170.46, 169.74, 169.36, 165.66, 73.35, 72.94, 70.43, 68302, 61.60, 61.56, 53.10, 53.00, 52.88, 52.15, 52.07, 52.05, 52.02, 51.63, 32.47, 32.31, 32.25, 31.91, 31.86, 31.72, 31.27, 31.12, 30.64, 30.56, 30.50, 29.43, 29.40, 29.34, 29.40, 29.34, 29.28, 29.24, 29.15, 29.05, 29.03, 28.99, 27.74, 25.35, 25.25, 25.20, 22.48, 20.51, 20.48, 20.42, 20.37, 13.91; HRMS calculated for [$C_{43}H_{72}N_3O_{14}$] [M+H] 854.5014, found 854.5020.

2.1.7. *2-(N-(2-(N-(4-(β-D-Glucopyranosylamino)succinyl))amino-D,L-dodecanoyl)-amino-D,L-dodecanoic acid* (***9a***)

Liposaccharide **8** (1.30 g, 1.52 mmol) was dissolved in methanol (30 ml) and the pH was adjusted to 12 using 1 M $NaOCH_3$ for 2 h, water (10 ml) was added and the pH was readjusted to 13. The solution was stirred at room temperature for an additional 12 h whereupon the reaction mixture was acidified using Amberlite resin IR-120 [H^+] until an acidic pH was obtained. The reaction mixture was filtered and the filtrate evaporated under vacuum. The residue was lyophilized from the acetonitrile:water (1:1) mixture to give a liposaccharide **9a** as a white powder.

2-(*N*-(2-(*N*-(4-(β-D-Glucopyranosylamino)succinyl))amino-D,L-dodecanoyl)-amino-D,L-dodecanoic acid **9a** (0.97 g, 1.45 mmol) was prepared in 95% yield; mp 155 °C; 1:1 mixture of diastereoisomers; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.18–4.98 (2H, m, 2CH (lipid)), 4.41–4.39 (2H, m, H-2and H-3 (glucose)), 3.70–60-3.56 (2H, m, H-4 and H-5 (glucose)), 3.48–3.43 (2H, m, H-$6_{a,b}$(glucose)), 2.83–2.79 (4H, m, 4OH (glucose)), 2.65–2.49 (4H, m, $2CH_2$ (succinic)), 1.75–1.72 (4H, m, β-$2CH_2$ (lipid)), 1.37–1.22 (32H, m, $2 \times 8CH_2$ (lipid)), 0.80 (6H, t, J = 6.8 Hz, $2CH_3$ (lipid)); ^{13}C NMR (500 MHZ, $CDCl_3$) δ 173.72, 173.64, 172.70, 172.46, 172.15, 172.15, 172.00, 171.96, 171.91, 171.80, 78.17, 78.14, 76.64, 76.62, 76.00, 71.24, 71.20, 68.57, 59.85, 52.11, 52.00, 50.23, 50.16, 30.23, 29.99, 29.30, 29.12, 28.80, 28.28, 27.87, 27.76, 27.61, 27.58, 27.53, 24.83, 24.67, 24.10, 24.06, 20.89, 11.60; HRMS calculated for [$C_{34}H_{62}N_3O_{10}$] [M+H] 672.4435, found 672.4441.

2.1.8. Sodium *2-(N-(2-(N-(4-(β-D-glucopyranosylamino)succinyl))amino-D,L-dodecanoyl)-amino-D,L-dodecanoate* (***9***)

The free acid **9a** (0.67 g, 1.00 mmol) was suspended in water (50 ml), $NaHCO_3$ (0.08 g, 1.00 mmol) was added and the mixture was sonicated. The reaction mixture was lyophilized to give a pure product **9** as a white powder.

Sodium 2-(*N*-(2-(*N*-(4-(β-D-glucopyranosylamino)succinyl))-amino-D,L-dodecanoyl)-amino-D,L-dodecanoate **9** (0.69 g, 1.00 mmol) was obtained in a quantitative yield; mp 180 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.18–4.98 (2H, m, 2CH (lipid)), 4.41–4.39 (2H, m, H-2 and H-3 (glucose)), 3.70-60–3.56 (2H, m, H-4 and H-5 (glucose)), 3.48–3.43 (2H, m, H-$6_{a,b}$(glucose)), 2.83–2.79 (4H, m, 4OH (glucose)), 2.65–2.49 (4H, m, $2CH_2$ (succinic)), 1.75–1.72 (4H, m, β-$2CH_2$ (lipid)), 1.37–1.22 (32H, m, $2 \times 8CH_2$ (lipid)), 0.80 (6H, t, J = 6.8 Hz, $2CH_3$ (lipid)); ^{13}C NMR (500 MHZ, $CDCl_3$) δ 173.72, 173.64, 172.70, 172.46, 172.15, 172.15, 172.00, 171.96, 171.91, 171.80, 78.17, 78.14, 76.64, 76.62, 76.00, 71.24, 71.20, 68.57, 59.85, 52.11, 52.00, 50.23, 50.16, 30.23, 29.99, 29.30, 29.12, 28.80, 28.28, 27.87, 27.76, 27.61, 27.58, 27.53, 24.83, 24.67, 24.10, 24.06, 20.89, 11.60; ESI-MS m/z: 672 [M+Na]; HRMS calculated for [$C_{34}H_{62}N_3O_{10}$] [M+Na] 672.4435, found 672.4441.

2.2. Isothermal titration calorimetry (ITC)

ITC experiments were conducted on a MicroCal VP-ITC calorimeter (MicroCal, Northampton, MA, USA) at 298 K in deionized water (pH 6.0) and data computed using Origin 5.0 software and VP viewer 2000 software. Aliquots of 10 μl of the concentrated solution of liposaccharide **9** (5.5 mM; 20 times of its critical aggregation concentration (CAC)) were mixed with water in the reaction cell (1.5 ml) under stirring at 300 rpm and 298 K. The duration of aliquot injections was 12 s and the time between two injections was 8 min. The peaks in the enthalpograms were integrated using Origin software to give the value for enthalpy as a function of total liposaccharide concentration in the cell. The enthalpy of aggregation of liposaccharide **9** was obtained from the difference between the initial and the final asymptotes of the sigmoidal curve. The CAC of compound **9** was obtained from the transition point of the enthalpy concentration profile.

2.3. Hemolytic assays

The potential liposaccharide interaction with erythrocyte membranes was investigated using hemolytic experiments. Hemolysis was used to quantify the membrane-damaging properties of liposaccharides **4** and **9**. Hemolytic assays were performed with the approval of the University of Queensland Ethics Committee (approval # 2009000661). Human erythrocytes were isolated from fresh heparin-treated blood collected from healthy adult volunteers by centrifugation at 3000 rpm for 15 min (Sigma 2-5 centrifuge, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The pellet was washed four times with an isotonic phosphate buffer saline (PBS) at pH 7.4, centrifuged at 3000 rpm for 15 min and resuspended in the same buffer. The erythrocyte pellet was diluted in PBS at pH 7.4 to a final concentration of 4% (v/v) erythrocytes. This stock solution was always freshly prepared and used within 24 h.

Liposaccharide derivatives **4** and **9** were prepared in PBS at different concentrations (0.1, 0.5, 1, 5 and 10 mM) and transferred to 96-well flat-bottom microtiter plates (TPP, Zurich, Switzerland). The plates were then incubated at 37 °C for 60 min on a Titramax 1000 Vibrating Shaker (Heidolph, Schwabach, Germany). Erythrocyte suspensions (100 μl) were added to each well on the plate and incubated for 60 min at 37 °C with constant shaking. After centrifugation at 3000 rpm for 15 min, the release of hemoglobin was determined by photometric analysis of the supernatant at 540 nm. Complete hemolysis was achieved by using 10 mM of sodium dodecyl sulfate (SDS) in PBS as a positive control (100%), while PBS was used as a negative control. Each experiment was performed in triplicate. The percentage of hemolysis was calculated according to the following formula:

$$\%\,\text{lysis} = \left[\frac{A_{\text{test}} - A_{\text{blank}}}{A_{100\%\text{lysis}} - A_{\text{blank}}}\right] \times 100$$

where A_{test} is the absorbance value of the hemoglobin released from erythrocytes treated with the test compounds; A_{blank} is the absorbance value of the hemoglobin released from erythrocytes treated with PBS buffer, and $A_{100\%\text{lysis}}$ is the absorbance value of

the hemoglobin released from erythrocytes treated with 10 mmol SDS in PBS solution (Pitarresi et al., 2007).

2.4. Cytotoxic assay

Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA) except for Hank's balanced saline solution, which was supplied by Sigma–Aldrich (Castle Hill, NSW, Australia). Tissue culture flasks (75 cm^2) were obtained from BD Bioscience (Franklin Lakes, NJ, USA). Caco-2 cells from the American Type Culture Collection (Rockville, MD, USA) were plated at a density of 4.0×10^3 cells/well in 96-well flat-bottomed microtiter plates and maintained in Dulbecco's Modified Eagle's Medium (DMEM; 25 mM D-glucose, 4 mM L-glutamine, 1 mM pyruvate) supplemented with 10% foetal bovine serum and 1% non-essential amino acids (0.1 mM) at 95% humidity and 37 °C in an atmosphere of 5% CO_2. The medium was changed every second day. After reaching 80% confluence, 100 U/ml penicillin and 100 mg/ml streptomycin were added into 100 μl of cell solution. The cells were allowed to grow for 2 days and then the medium was changed.

The toxicity of anionic liposaccharides **4** and **9** on Caco-2 cells was evaluated by MTT that was obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). After washing with PBS, the cells were incubated with 100 μl of test samples and controls in DMEM. DMEM alone was used as a negative control and SDS (0.1 mM) as a positive control. The liposaccharides **4** and **9** in DMEM media were incubated at 37 °C for 12 h with a Caco-2 monolayer at various concentrations (0.1, 0.5, 1, 5, and 10 mM). After incubation, the cell viability was assessed using a colorimetric MTT assay. MTT was prepared at 5 mg/ml in PBS and 20 μl was applied to each well. After 3 h incubation of MTT with cells, 100 μl of dimethyl sulfoxide (DMSO) was added in order to solubilize the MTT-formazan product. The cells were subcultured using 0.2% ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin (Motlekar et al., 2005). Absorbance at 590 nm was measured with a microplate reader. The cell viability was expressed as the percentage of absorbance of the tested compounds relative to the positive control.

2.5. Partition coefficient studies

The partition coefficients (between *n*-octanol and water) of tobramycin with liposaccharides **4** and **9** at 1:1 and 1:7 molar ratios were determined. Each phase was saturated with the other phase by an overnight equilibration before the experiments. Different standard solutions of tobramycin were prepared and a standard calibration curve was prepared. An equal volume (0.5 ml) of saturated *n*-octanol with water was added and the pH was adjusted to 7.4 by 1% HCl or 1% sodium hydroxide (NaOH). Blank samples containing tobramycin only were also used. The mixtures were shaken for 3 h at room temperature and then separated by centrifugation at 3000 rpm for 30 min. After separation, the drug concentrations in the aqueous phases were determined by LC–MS/MS. The amount of the drug disappearing from the aqueous phase was considered to be partitioned into the *n*-octanol phase.

The apparent *n*-octanol/water partition coefficient was calculated from the concentration of tobramycin in the aqueous layer before and after the addition of *n*-octanol. All experiments were conducted in triplicate. The partitioning coefficient was calculated using the following equation:

$$\text{Log}\, p_{ow} = \log \frac{a_o}{a_w}$$

where a_o and a_w were the concentrations of tobramycin in the *n*-octanol and in the aqueous phases, respectively.

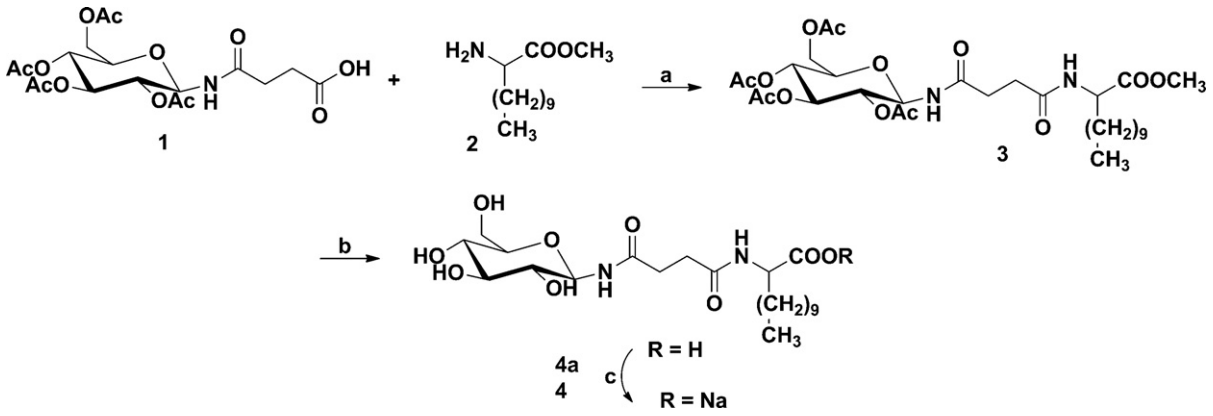
3. Results and discussion

3.1. Anionic liposaccharide derivatives

D-Glucose was peracetylated by acetic anhydride, followed by bromination of the carbohydrate anomeric position using hydrogen bromide in acetic acid (Zhang et al., 2008). The method for the β-D-glucopyranosyl azide synthesis using peracetylated α-D-glucopyranosyl bromide with sodium azide in the mixture of acetone and water (Ibatullin and Shabalin, 2000) was found to be more effective (higher yield, lower cost, ease of synthesis) than the method (Harrison et al., 1994) using *tetra*-butyl ammonium hydrogen sulfate in DCM/aqueous $NaHCO_3$ mixture. Therefore, the peracetylated α-D-glucopyranosyl bromide was reacted with sodium azide in a mixture of acetone and water (4:1) which gave the corresponding β-D-glucopyranosyl azide with an 86% yield after re-crystallization from hot ethanol (Ibatullin and Shabalin, 2000). The azide was reduced to amine by hydrogenation catalyzed by 10% palladium on carbon. To avoid migration of the acetyl group onto the reactive amine (Skwarczynski and Kiso, 2007), coupling of amine to succinic anhydride in dry DCM or tetrahydrofuran (THF) with the catalysis of dimethyl aminopyridine (DMAP) (Moyle et al., 2007) was performed in situ, immediately after the azide reduction. It was important to use only one mole equivalent of succinic anhydride in order to overcome the difficulties observed during the separation of the resulting carbohydrate derivative **1** from succinic acid (Scheme 1). Unreacted peracetylated glucosyl amine was easily removed by acid–base wash. 2-Amino dodecanoic acid was synthesized as reported previously (Gibbons et al., 1990). Different protecting groups (cyclohexyl, benzyl, methyl) were applied for the synthesis of protected LAA. Both the cyclohexyl and the benzyl groups were difficult to introduce/remove as it needed drastic conditions for a long period of time (Hwang et al., 2007). Methylation of the 2-amino dodecanoic acid was preferred because of the ease of esterification and its subsequent deprotection performed by aqueous NaOH to prepare protected LAA **2**. The carboxyl group was esterified using thionyl chloride in methanol to give 2-amino dodecanoic acid methyl ester hydrochloride salt. The excess of thionyl chloride was removed by vacuum followed by neutralization with aqueous $NaHCO_3$ to produce methylated LAA (C12) **2** (Peuralahti et al., 2006). Glucosyl derivative **1** was coupled to the methylated LAA **2** using HBTU and DIPEA to form a liposaccharide which was purified by flash chromatography to give a compound **3** in 70% yield (Scheme 1).

The acetyl groups on the carbohydrate derivative **3** were removed by Zemplén deacetylation using 1 M $NaOCH_3$ in methanol at pH 13. Water was added to the reaction mixture to hydrolyze the methyl derivative. The reaction mixture was stirred for an additional 12 h, acidified using acidic resin IR-120 $[H]^+$, filtered, evaporated under vacuum and lyophilized from acetonitrile:water (1:1) to give a liposaccharide **4a** with free carboxylic acid in 89% yield. To facilitate ion-pairing of the formed liposaccharide with the positively charged drug and to increase the aqueous solubility of the final complex, the sodium derivative **4** was synthesized by sonication of the mixture of free acid **4a** with one equivalent of aqueous $NaHCO_3$. The sodium derivative **4** was obtained after lyophilization in quantitative yield as a white powder.

Lipid derivative **6** was prepared by coupling methylated LAA **2** to Boc-protected LAA **5** as shown in Scheme 2. LAA **5** was synthesized as previously described for LAA **2** followed by Boc protection of the N^{α}-amine using Boc_2O (Gibbons et al., 1990). LAAs **2** and **5** were conjugated using HBTU/DIPEA to give, after column flash chromatography, Boc-protected lipid moiety **6** in 74% yield. The Boc group was removed using TFA in DCM and thus the corresponding di-LAA was obtained in a quantitative yield. The trifluoroacetate salt was neutralized by aqueous $NaHCO_3$, dried over $MgSO_4$,



Scheme 1. Glucopyranosyl succinate coupling to methylated LAA to form liposaccharide **4** containing one LAA. (a) HBTU, DIPEA, DCM, 24 h; (b) (i) 0.1 M $NaOCH_3$, CH_3OH, 2 h; (ii) H_2O, 12 h; (iii) Amberlite IR-120 (H^+); (c) $NaHCO_3$. HBTU: *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetra-methyl-uronium-hexafluoro-phosphate; DIPEA: diisopropylethyl amine; DCM: dichloromethane; $NaOCH_3$: sodium methoxide; CH_3OH: methanol; IR: ion exchange resin; $NaHCO_3$: sodium bicarbonate.

filtered and evaporated under vacuum to give the lipidic compound **7**. Di-LAA **7** was further conjugated to the glucopyranosyl succinate **1** using HBTU/DIPEA in dry DCM providing a 65% yield of liposaccharide **8** after a column flash chromatography. Deacetylation, hydrolysis and sodium salt formation were performed as described above. The reaction mixture was acidified using Amberlite resin, filtered, evaporated under vacuum and lyophilized from the acetonitrile:water (1:1) to get the di-liposaccharide **9a** and its sodium salt **9** in 95% and quantitative yields, respectively. All the structures were confirmed by ^1H, ^{13}C NMR and mass spectroscopy (MS).

3.2. Aggregation of anionic liposaccharides

The liposaccharides formed aggregates in aqueous solution what could affect their interactions with the co-administered drug. The determination of CAC was therefore an important step in the prediction of the type of interaction that could have arisen during the association of these liposaccharides with a drug candidate. Van Os method was used to estimate CAC of the compounds, where the cumulative enthalpy was plotted as a function of liposaccharide concentration (van Os et al., 1991). The enthalpy data obtained from ITC measurements of liposaccharide **4** were low ($\leq$0.5 kJ mol^{-1}) and the CAC of this compound could not be accurately estimated. Either liposaccharide **4** did not aggregate or the formed aggregates were not stable in water to allow ITC results.

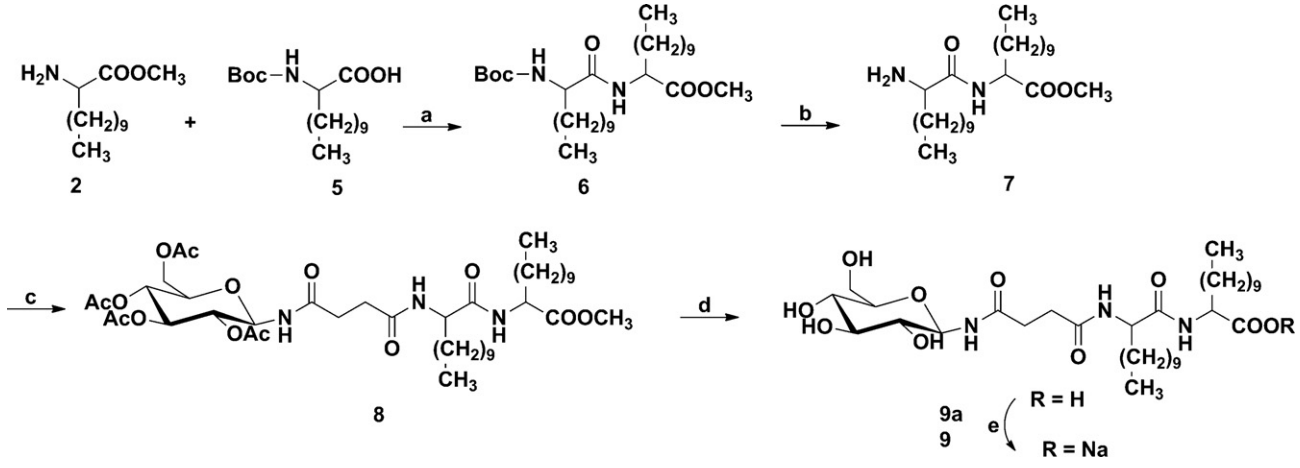
The plots of the calorimetric titration of liposaccharide **9** showed a change of slope around the CAC (Fig. 2). The CAC value of this compound was calculated by selecting data above and below the CAC, fitting them into a linear regression and taking their line-line intersection as the CAC = 0.275 ± 0.010 mM (Fig. 2a and b).

The Gibbs free energy of aggregation (ΔG_{agg}) can be calculated by the following equation:

$$\Delta G_{agg} = RT \ln X_{agg}$$

where R is the gas constant (8.314 J K^{-1} mol^{-1}), T is the absolute temperature = 298 K, and X_{agg} is the CAC value in moles. ΔG_{agg} for liposaccharide **9** was calculated to be −31.46 kJ mol^{-1}.

The steep decrease in the exothermic enthalpy change (ΔH_{agg}) of liposaccharide **9** observed at its CAC (0.275 mM) could be attributed to the fact that the concentration of liposaccharide **9** in the reaction cell exceeded the CAC and the injected aggregates no longer disaggregated (Fig. 2b). The relatively small exothermic ΔH_{agg} measured at high concentrations (>0.6 mM) could be explained by dilution effects. ΔH_{agg} of compound **9** (determined from the inflexion point in the heat change versus surfactant concentration curve) was −7.55 kJ mol^{-1} (Fig. 2c). The negative enthalpy value indicating an exothermic process can be attributed to increased van der Waals and hydrophobic



Scheme 2. Synthesis of liposaccharide **9** containing two LAAs. (a) HBTU, DIPEA, DCM, 24 h; (b) (i) TFA: DCM (1:1), 1 h; (ii) $NaHCO_3$; (c) glucopyranosyl succinate **1**, HBTU, DIPEA, DCM, 24 h; (d) (i) 0.1 M $NaOCH_3$, CH_3OH, 2 h; (ii) H_2O, 12 h; (iii) Amberlite IR-120 (H^+); (e) $NaHCO_3$. HBTU: *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetra-methyl-uronium-hexafluoro-phosphate; DIPEA: diisopropylethyl amine; DCM: dichloromethane; TFA: trifluoroacetic acid; $NaHCO_3$: sodium bicarbonate; CH_3OH: methanol; IR: ion exchange resin.

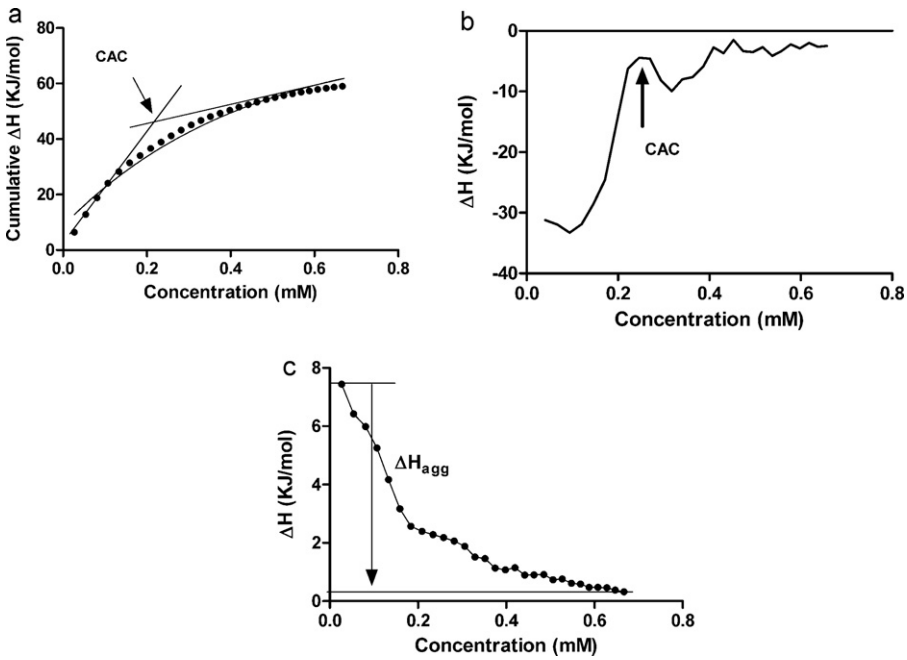


Fig. 2. Determination of the enthalpy of aggregation and CAC of 4 mM liposaccharide **9** at 298 K. (a) Determination of the CAC through cumulative enthalpy versus concentration of compound **9**. (b) First derivative of the heat of reaction. The CAC is determined from the maximum of this curve. (c) Heat of reaction versus concentration of liposaccharide **9**. CAC: critical aggregation concentration; ΔH: enthalpy change.

interactions, which contributed to aggregate formation (Bai et al., 2001). Similar effect, high exothermic ΔH_{agg} at low concentrations, was observed in another study where the disaggregation of the aggregates which entered the reaction cell and were diluted to the concentrations below the CAC (Thongngam and McClements, 2004). ΔG_{agg} for liposaccharide **9** was $-31.46\ kJ\ mol^{-1}$ and showed that favorable changes occurred during the aggregation process leading to the formation of stabilized particles in the aqueous environment.

The formation of larger aggregates was previously observed with more lipophilic compounds (Coles et al., 2011). Transmission electron microscopy (TEM) micrographs of liposaccharide **9** showed aggregation behavior at the CAC. Liposaccharide **9** formed poly-dispersed aggregates with size around 200–300 nm (Fig. 3). These results correlate with the size determined by dynamic light scattering (data not shown), where a highly poly-dispersed size distribution of compound **9** was observed.

3.3. Hemolytic assay

The release of hemoglobin was used to quantify the membrane-damaging properties of the liposaccharides. Erythrocytes were treated with PBS and SDS to obtain values corresponding to 0 and 100% of lysis, respectively. The erythrocytes were incubated for 1 h with five different concentrations (0.1, 0.5, 1, 5 and 10 mM) of liposaccharides **4** or **9** to measure hemolytic lysis. Both compounds **4** and **9** caused almost no hemolysis (≤20%) (Amin and Dannenfelser, 2006) to human red blood cells (hRBCs) at lower concentrations (≤1 mM) used for oral administration (Fig. 4). However, both liposaccharides **4** and **9** caused a significant lysis (over 80%) of hRBCs at higher concentrations (≥5 mM). The small increase in the hemolysis was observed in the case of the more lipophilic and surfactant-like liposaccharide **9** even though the difference is not significant. These results correlate with our previously reported data (Abdelrahim et al., 2009).

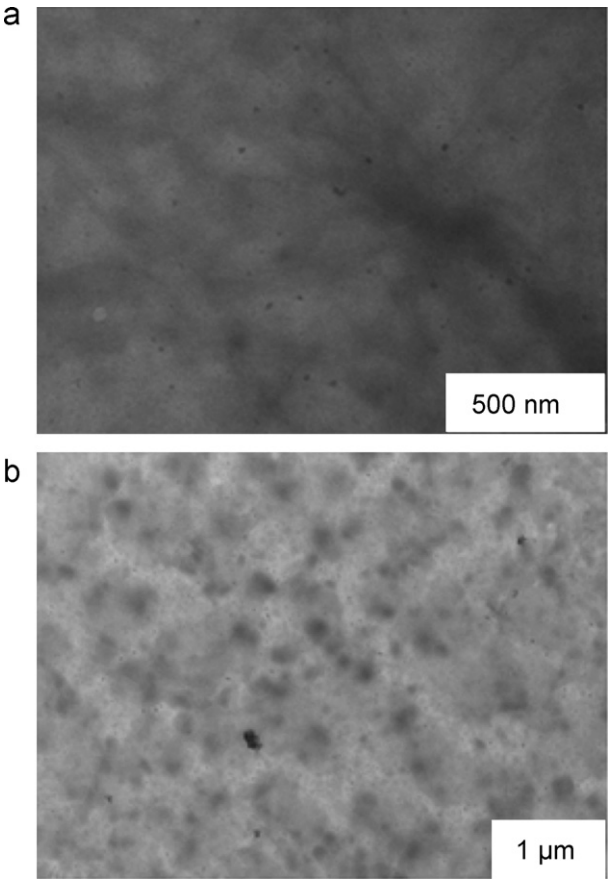


Fig. 3. The transmission electron microscopy images of liposaccharide **9** at CAC; scale: (a) 500 nm; (b) 1 μm. CAC: critical aggregation concentration.

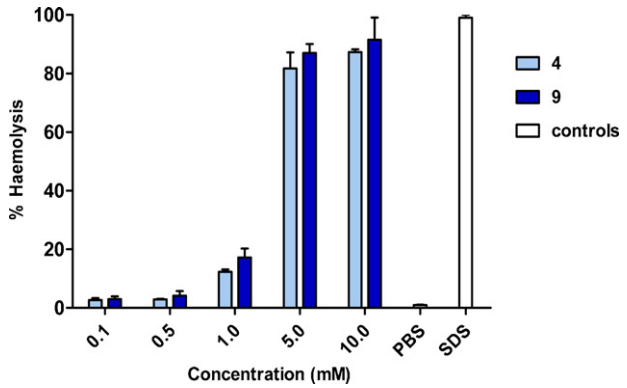


Fig. 4. Hemolytic effect of liposaccharide derivatives **4** and **9** in PBS at concentrations 0.1–10 mM measured against hRBCs. All measurements were made in triplicate and are shown as a mean ± SD. PBS: phosphate buffer saline; hRBCs: human red blood cells; SD: standard deviation.

3.4. Cytotoxic assay

The effect of different concentrations of anionic liposaccharide derivatives (0.1, 0.5, 1, 5 and 10 mM) on Caco-2 (human epithelial colorectal adenocarcinoma) cells was evaluated by MTT assay. The contact time between the liposaccharide derivatives and Caco-2 cells was 12 h as reported elsewhere (Sethia and Squillante, 2004). To obtain the values corresponding to 0 and 100% of cell viability, Caco-2 cell monolayers were incubated with SDS and DMEM, respectively. The results shown in Fig. 5 indicated that liposaccharide derivatives **4** and **9** did not cause more than 40% decrease in Caco-2 cell viability at all tested concentrations including 5 and 10 mM.

Interestingly, the liposaccharide derivatives **4** and **9** had different effects on different cells. The results from MTT assay showed that the liposaccharides **4** and **9** caused less than 40% decrease in epithelial Caco-2 cell viability at all tested (even up to 10 mM) concentrations (Fig. 5). This result was unexpected since the hemolytic activity assay showed over 80% hemolysis of hRBCs at concentrations of 5 and 10 mM.

3.5. Partition coefficient studies

Model drug tobramycin was formulated with anionic liposaccharides **4** and **9** in two molar ratios; 1:1 and 1:7. The partitioning coefficients of tobramycin with or without liposaccharides **4** and **9** were determined between *n*-octanol and water. Lipophilicity

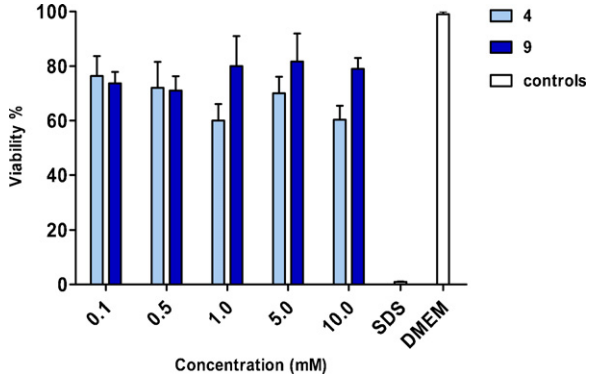


Fig. 5. Mitochondrial dehydrogenase activity shown as a percentage of viability of Caco-2 cells after treatment with liposaccharide derivatives 4 and 9 at various concentrations (0.1–10 mM). All measurements were made in triplicate and shown as a mean ± SD. Caco-2: human epithelial colorectal adenocarcinoma; SD: standard deviation.

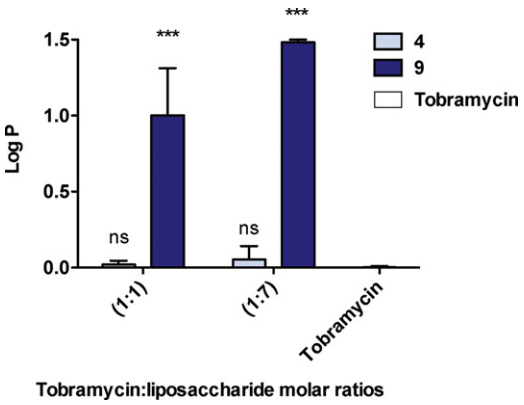


Fig. 6. Partitioning studies of tobramycin with liposaccharide derivatives 4 and 9 at molar ratios of 1:1 and 1:7 between *n*-octanol/water. Values are expressed as the log *P* of tobramycin. All measurements were made in triplicate and shown as a mean ± SD. Log *p*: partition coefficient; SD: standard deviation. Statistical analysis was performed using a one-way ANOVA followed by the Dunnett's post hoc test (ns, $p > 0.05$; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

was expressed in log *p* value that is one of the most important physico-chemical characteristics in predicting and interpreting membrane permeability during passive drug transport (Mrestani et al., 2004). The liposaccharide **4** containing one lipid moiety did not increase partitioning of the complex significantly (both log *p* values were below 0.05 indicating low lipophilicity and partitioning of the drug/liposaccharide **4** aggregate) however a significant increase of partition coefficient was observed for tobramycin in the presence of the liposaccharide **9** (containing two lipid moieties) at both 1:1 and 1:7 molar ratios; $\log p = 1.001 \pm 0.310$ and 1.482 ± 0.017, respectively (Fig. 6). Moreover, the log *p* values of the tobromycin:liposaccharide 1:7 molar ratio were 1.5 fold higher than those of 1:1 molar ratio.

3.6. Statistical analysis

Pharmacokinetic parameters of different formulations were compared using a one-way ANOVA test. When the differences in the means were significant, Dunnett's post hoc test was conducted using Newman–Keuls multiple comparison (GraphPad Prism, version 4.0; GraphPad Software, San Diego, CA, USA). Differences in *p* values < 0.05 were considered statistically significant.

4. Conclusion

Anionic liposaccharide derivatives **4** and **9** containing one and two LAAs were successfully synthesized to improve the gastrointestinal absorption of charged and hydrophilic drug tobramycin sulfate. ITC data of liposaccharide **4** were so low that the CAC of this compound could not be accurately estimated. The CAC of two LAAs containing liposaccharide **9** was determined by ITC to be 0.275 ± 0.010 mM and its thermodynamic parameters including ΔH_{agg} and ΔG_{agg} were calculated. Liposaccharide **9** at the CAC formed aggregates of 200–300 nm size as observed by TEM. Both anionic liposaccharides **4** and **9** showed only slight hemolytic activity (≤20%) of hRBCs at concentrations commonly used for oral administration of drugs with absorption enhancers (≤1 mM). The viability of Caco-2 cell monolayers after the application of liposaccharides (up to 10 mM concentrations) onto the cells did not decrease below 60%. These compounds were therefore considered to be safe for drug delivery. To characterize the hydrophobic/hydrophilic properties of the tobramycin/liposaccharide complex, partition coefficients were measured in the *n*-octanol/water system using two different molar

ratios. Tobramycin alone exhibited, as expected, a very small partition coefficient ($\log p = 0.006 \pm 0.003$). Combinations of tobramycin with anionic liposaccharide **9** caused a considerable improvement in tobramycin partitioning at both (1:1 and 1:7) molar ratios ($\log p = 1.001 \pm 0.310$ and 1.482 ± 0.017, respectively).

Our study showed that the liposaccharide **9** was safe and could be effective absorption enhancer of hydrophilic, poorly orally available drugs.

Acknowledgements

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