

Supplementary Materials: Bi-Functional Alginate Oligosaccharide–Polymyxin Conjugates for Improved Treatment of Multidrug-Resistant Gram-Negative Bacterial Infections

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1. Methods

NMR Spectroscopy

Samples (10 mg) were dissolved in 99.9 atom % D₂O (600 μL) and analysed by NMR at 25 °C on a Bruker AVIII-HD 800 spectrometer equipped with CP-TCI 5 mm cryoprobe (Bruker BioSpin AG; Fällanden, Switzerland). Diffusion-ordered spectroscopy (DOSY) was used to measure the diffusion of the OligoG conjugate products. A 2D DOSY was measured using a Bruker BioSpin stimulated echo pulse sequence with bipolar gradients (STEBPGP). Gradient pulses of 2 ms duration (δ) and 32 different strengths varying linearly from 0.03 to 0.57 Tm⁻¹ were applied and the diffusion delay (Δ) was set to 80 ms. The spectra were recorded using the TopSpin software (version 3.5pl7) and analysed with the TopSpin software (version 4.0.7) (Bruker BioSpin AG; Fällanden, Switzerland).

2. Results

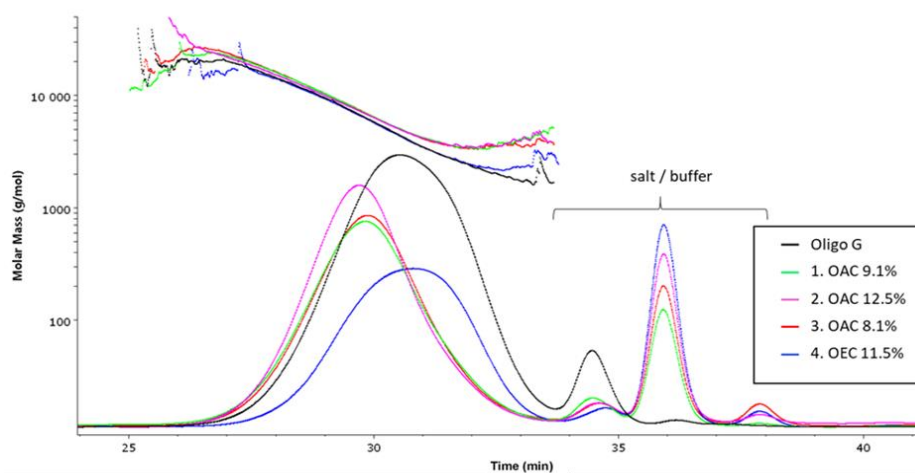


Figure S1. Size exclusion chromatography with multi-angle light scattering detection (SEC-MALS) analysis of OligoG-conjugates (three different batches of OligoG-A-colistin (OAC) and one batch of OligoG-E-colistin (OEC)), showing overlaid refractive index chromatograms and corresponding Mw-time calibration lines. The injected mass was 250 μg for all samples. Abbreviations: A, amide; E, ester.

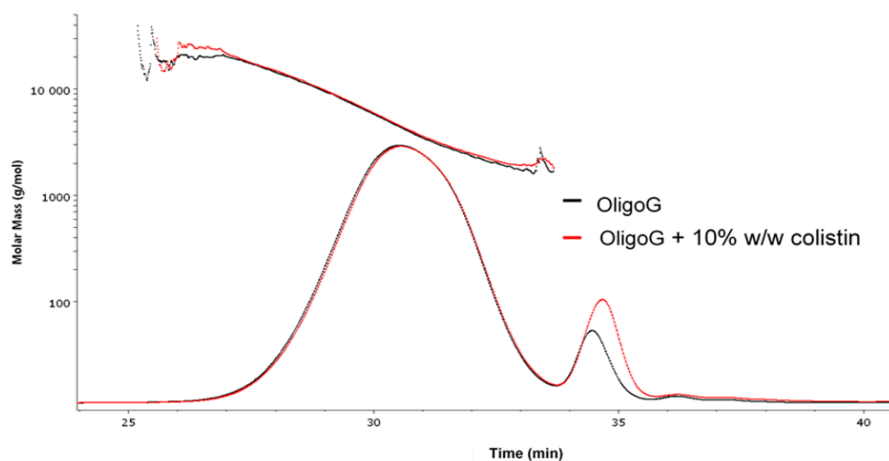


Figure S2. SEC-MALS analysis of OligoG in the absence and presence of 10% *w/w* colistin, showing that they do not form strong complexes since the elution profile is identical.

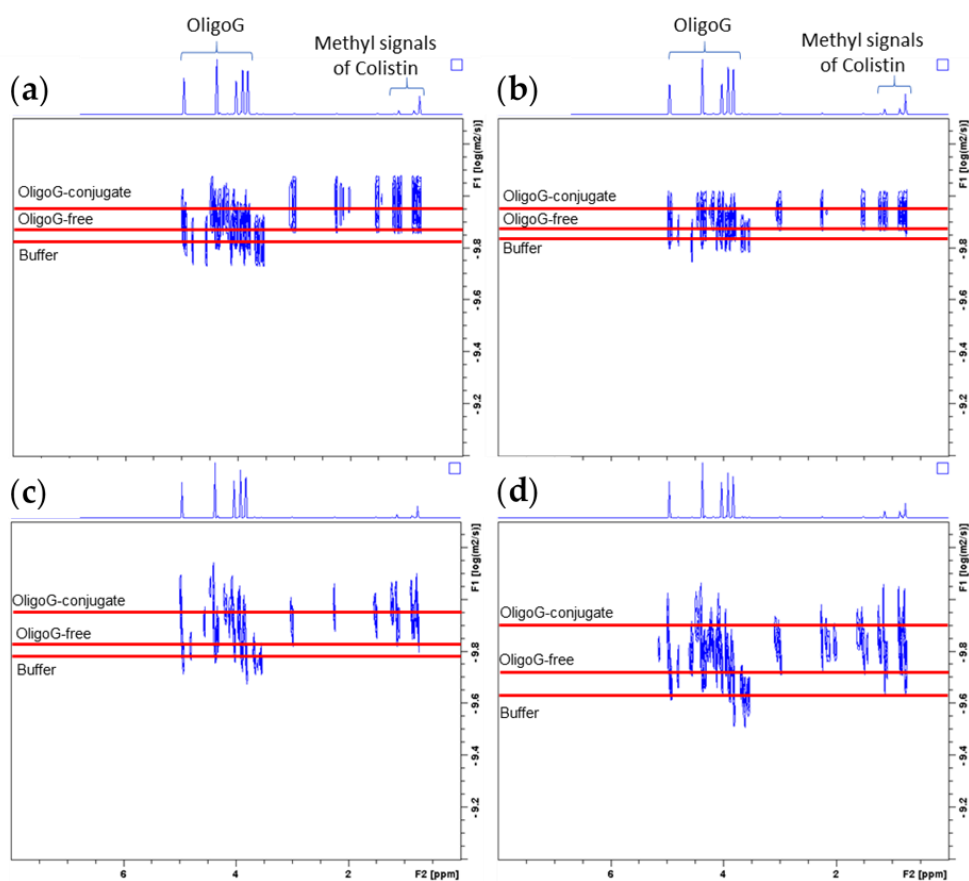


Figure S3. Diffusion-ordered spectroscopy (DOSY) of (a-c) three different batches of OligoG-A-colistin conjugates and (d) one batch of OligoG-E-colistin conjugate. The assignment of the unique signals for OligoG and colistin is indicated at the top of each panel and the red lines indicate the average diffusion coefficients of the molecules.

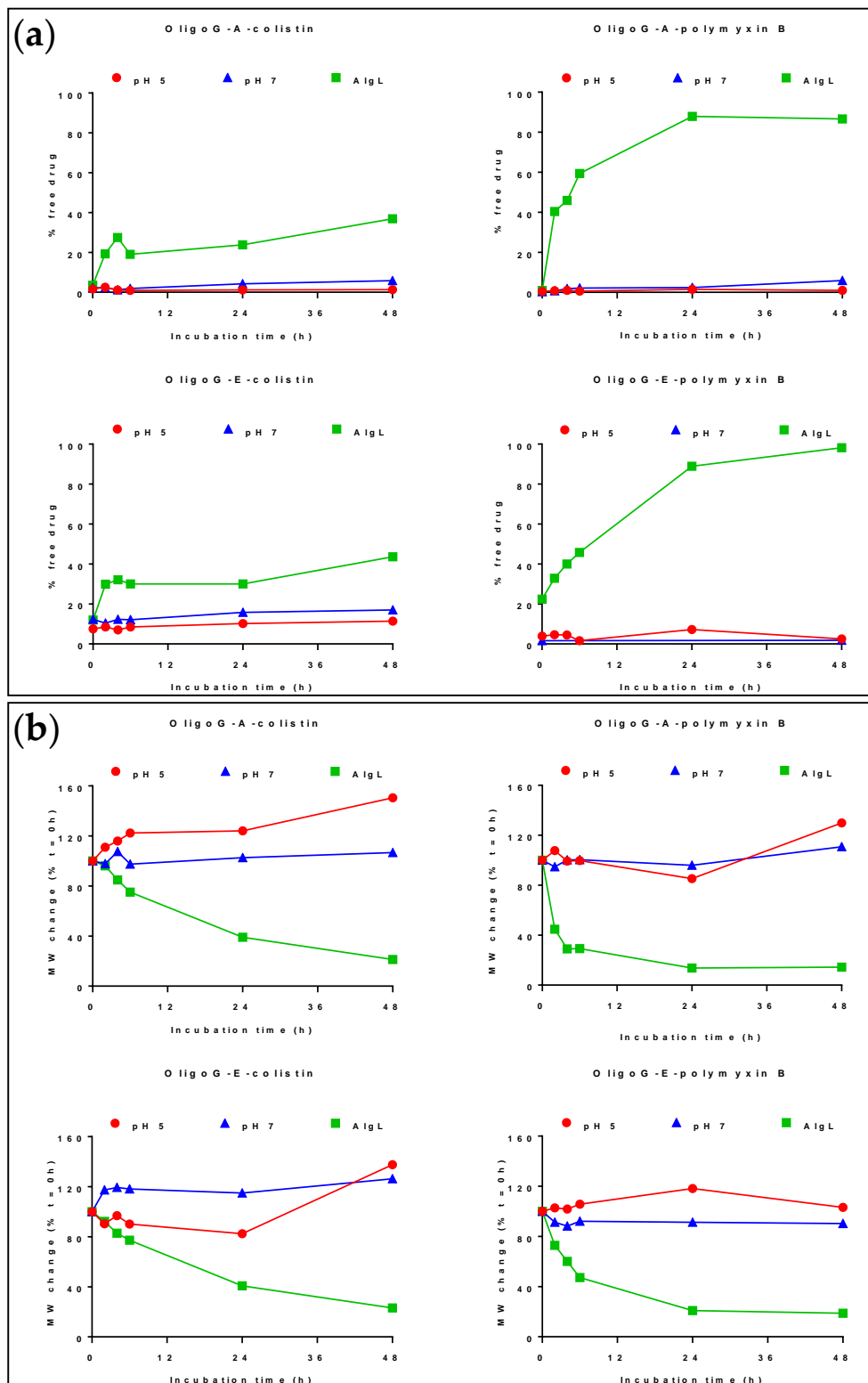


Figure S4. Drug release of OligoG-polymyxin conjugates in phosphate buffered saline (PBS) at pH 5, pH 7 or pH 7 containing alginate lyase (AlgL). (a) Content of free polymyxin and (b) change in molecular weight were determined by fast protein liquid chromatography (FPLC) and size exclusion chromatography with refractive index detection (SEC-RI), respectively, over 48 h incubation. Abbreviations: A, amide; E, ester.

Table S1. Gram-negative bacterial isolates used for characterisation of OligoG-polymyxin conjugates.

Bacterial Isolate	Genotype	Origin
<i>P. aeruginosa</i> R22	VIM-2	China
<i>P. aeruginosa</i> MDR 301	VIM-2	Poland
<i>P. aeruginosa</i> NH57388A		Denmark
<i>P. aeruginosa</i> NCTC 10662		Reference strain
<i>K. pneumoniae</i> KP05 506	NDM-1	India
<i>K. pneumoniae</i> IR25	NDM-1	India
<i>A. baumannii</i> MDR ACB	MDR	Libya
<i>A. baumannii</i> 7789	MDR	United Kingdom
<i>E. coli</i> AIM-1	AIM-1	Australia
<i>E. coli</i> IR57	NDM-1	India
<i>E. coli</i> 5702		United Kingdom
<i>E. coli</i> NCTC 10418		Reference strain
<i>E. coli</i> PN21	<i>mcr-1</i>	Thailand
<i>E. coli</i> PN25	<i>mcr-1</i>	Thailand
<i>E. coli</i> PN26		Thailand
<i>E. coli</i> ATCC 25922		Reference strain

VIM-2, carbapenem-hydrolyzing metallo- β -lactamase; NDM-1, New Delhi metallo- β -lactamase; AIM-1, metallo- β -lactamase; MDR, multidrug resistant; *mcr-1*, plasmid-encoded colistin resistance.

Table S2. Physicochemical characteristics and batch details of OligoG-polymyxin conjugates used in this study.

Tested Compound	Mw (g/mol) (PDI) by SEC-RI*	Mw (g/mol) (PDI) by SEC-MALS	Drug Content (% w/w)	Molar Ratio (per Colistin)	Conjugated NH ₂ per Molecule	Free Drug (%)	Experiments Performed					
							Physicochemical	Cytotoxicity	Antimicrobial Activity	Growth Curves	Biofilm Formation	PK-PD
OligoG-A-colistin	25,500 (2.6)	12,300 (1.4)	8.7	4.6	2.8	5.7	x	x	x	x		
OligoG-A-colistin	22,500 (2.3)	9100 (1.3)	8.8	4.6	2.7	1.5	x		x	x	x	
OligoG-A-colistin	24,500 (2.3)	8300 (1.3)	9.1	4.4	3.6	2.5	x		x			
OligoG-A-colistin	27,000 (2.5)	8200 (1.2)	12.5	3.1	3.3	2.4	x		x			
OligoG-A-colistin	24,500 (2.2)	8200 (1.3)	8.1	5.0	4.6	4.0	x					x
OligoG-E-colistin	14,500 (2.3)	5900 (1.2)	12.9	3.0	N/A	2.0	x	x	x	x		
OligoG-E-colistin	16,500 (2.2)	5200 (1.3)	11.5	3.4	N/A	3.5	x		x	x	x	
OligoG-E-colistin	20,000 (3.1)	ND	8.3	4.9	N/A	2.7	x					x
OligoG-A-polymyxin B	23,000 (2.7)	12,800 (1.5)	8.0	5.1	1.9	1.6	x	x	x	x		
OligoG-A-polymyxin B	23,500 (2.2)	9100 (1.3)	6.1	6.8	2.0	1.6	x		x	x		
OligoG-E-polymyxin B	15,500 (2.1)	6200 (1.2)	7.0	5.9	N/A	2.7	x	x	x	x		

Abbreviations: A, amide; E, ester; SEC-MALS, size exclusion chromatography with multi-angle light scattering detection; SEC-RI, size exclusion chromatography with refractive index detection; PDI, polydispersity index; ND, not determined; N/A, not applicable. *Molecular weight was estimated relative to pullulan standards.

Table S3. Weight and number average molecular weights of OligoG and OligoG-colistin conjugates.

Tested Compound	Drug Content (% w/w)	Mn (kDa)	Mw (kDa)	Molar Ratio (per Colistin)*	Mn (kDa)*	Mw (kDa)*
OligoG	N/A	3.9	5.5	N/A	N/A	N/A
OligoG-A-colistin	9.1	6.6	8.3	2	4.3	8.3
OligoG-A-colistin	12.5	6.6	8.2	1.4	4.4	9.4
OligoG-A-colistin	8.1	6.4	8.2	2.5	4.2	8.0
OligoG-E-colistin	11.5	4.0	5.2	1.7	4.3	9.1

*Expected values assuming 100% conjugation calculated from the Mn and Mw values of OligoG and the % w/w of each sample.

Abbreviations: A, amide; E, ester; N/A, not applicable.

Table S4. Selectivity index (SI) values of OligoG-polymyxin conjugates against a range of Gram-negative bacterial pathogens.

Isolate	SI Value					
	Colistin Sulphate	Polymyxin B	OligoG-E-Colistin	OligoG-E-Polymyxin B	OligoG-A-Colistin	OligoG-A-Polymyxin B
<i>P. aeruginosa</i> R22	52	44	57	128	121	75
<i>P. aeruginosa</i> MDR 301	52	22	114	64	242	150
<i>P. aeruginosa</i> NH57388A	104	44	228	128	484	299
<i>P. aeruginosa</i> NCTC 10662	208	175	228	128	242	75
<i>K. pneumoniae</i> KP05 506	208	88	456	128	1936	598
<i>K. pneumoniae</i> IR25	413	88	456	256	242	75
<i>A. baumannii</i> MDR ACB	52	88	228	508	242	150
<i>A. baumannii</i> 7789	104	88	456	64	1936	598
<i>E. coli</i> AIM-1	208	22	228	64	1936	150
<i>E. coli</i> IR57	839	175	1839	508	3841	1196
<i>E. coli</i> 5702	208	44	114	128	968	75
<i>E. coli</i> NCTC 10418	3	1	4	4	8	9
<i>E. coli</i> PN21	3	3	7	8	8	9
<i>E. coli</i> PN25	208	88	228	128	484	598
<i>E. coli</i> PN26	104	22	57	64	242	19
<i>E. coli</i> ATCC 25922	52	44	57	128	121	75
Mean	184	66	313	154	862	272

Selectivity index (SI) = IC₅₀ (µg/mL)/MIC (µg/mL). Mean SI is shown in **bold**. Abbreviations: A, amide; E, ester.

Table S5. Microbiological efficacy (MICs) of OligoG-colistin conjugates in the presence of alginate lyase in Mueller–Hinton (MH) broth or after pre-incubation with alginate lyase against Gram-negative bacterial pathogens.

Isolate	Tested Compound MIC ($\mu\text{g/mL}$ Drug Base) at Indicated Alginate Lyase Concentration (U/mL)								
	OligoG-A-Colistin			OligoG-E-Colistin			Colistin Sulphate		
	0	1	10	0	1	10	0	1	10
Alginate Lyase in MH Broth									
<i>P. aeruginosa</i> MDR 301	1	1	0.25	0.5	1	0.25	0.063	0.063	0.25
<i>K. pneumoniae</i> KP05 506	0.063	0.125	0.063	0.125	0.25	0.063	0.008	0.063	0.008
<i>E. coli</i> AIM-1	0.00002	0.0001	0.0002	0.000008	0.0002	0.0002	0.00006	0.00006	0.0002
<i>A. baumannii</i> 7789	0.063	0.063	0.008	0.125	0.125	0.125	0.002	0.004	0.004
Pre-incubation with Alginate Lyase									
<i>P. aeruginosa</i> MDR 301	1	0.25	0.25	0.5	0.125	0.125	0.063	0.063	0.125
<i>K. pneumoniae</i> KP05 506	0.063	0.008	0.004	0.125	0.063	0.063	0.008	0.004	0.004
<i>E. coli</i> AIM-1	0.00002	0.001	0.0002	0.000008	0.001	0.001	0.00006	0.001	0.0002
<i>A. baumannii</i> 7789	0.063	0.016	0.004	0.125	0.063	0.063	0.002	0.002	0.002

Abbreviations: A, amide; E, ester.

Table S6. Microbiological efficacy (MICs) of polymyxins and antibiotic conjugates in the absence and presence of mucin against Gram-negative bacterial pathogens.

Tested Compound ↓	Mucin (%) →	MIC ($\mu\text{g/mL}$ Drug Base)											
		<i>P. aeruginosa</i> R22			<i>A. baumannii</i> MDR ACB			<i>K. pneumoniae</i> IR25			<i>E. coli</i> IR57		
		0	0.2	2	0	0.2	2	0	0.2	2	0	0.2	2
OligoG-A-colistin		4	4	64	0.125	4	64	0.125	8	>128	0.063	2	8
OligoG-E-colistin		1	16	64	1	16	64	0.063	32	>128	0.063	4	64
0.2% OligoG + colistin		0.25	2	32	ND	ND	ND	ND	ND	ND	<0.063	2	4
2% OligoG + colistin		1	4	32	ND	ND	ND	ND	ND	ND	0.25	0.5	2
Colistin sulphate		0.5	2	16	0.5	1	16	0.063	1	64	0.125	1	2
OligoG-A-polymyxin B		2	64	>128	4	16	128	2	16	>128	2	16	32
OligoG-E-polymyxin B		0.5	2	32	0.25	1	16	0.125	2	128	0.125	0.5	1
0.2% OligoG + polymyxin B		0.5	4	64	ND	ND	ND	ND	ND	ND	0.125	2	4
2% OligoG + polymyxin B		1	4	32	ND	ND	ND	ND	ND	ND	<0.063	0.5	2
Polymyxin B		0.25	4	64	0.125	1	16	0.125	4	64	0.5	1	4

Abbreviations: A, amide; E, ester; ND, not determined.

Table S7. Comparison of the effect of growth medium (AS medium and MH broth) on antimicrobial activity (MIC determinations) of polymyxins and antibiotic conjugates.

Tested compound ↓	Medium →	MIC (µg/mL Drug Base)											
		<i>P. aeruginosa</i> MDR 301		<i>P. aeruginosa</i> NH57388A		<i>K. pneumoniae</i> KP05 506		<i>E. coli</i> IR57		<i>E. coli</i> 5702		<i>A. baumannii</i> 7789	
		AS	MH	AS	MH	AS	MH	AS	MH	AS	MH	AS	MH
OligoG-A-colistin		4	1	2	0.5	2	0.125	0.125	0.125	0.25	0.063	2	0.125
OligoG-E-colistin		4	0.5	1	0.25	1	0.125	0.063	0.25	0.125	0.031	1	0.125
Colistin sulphate		2	0.5	1	0.25	1	0.125	0.125	0.125	0.125	0.031	1	0.25
OligoG-A-polymyxin B		16	2	4	1	8	0.5	0.5	2	1	0.25	4	0.5
OligoG-E-polymyxin B		8	0.5	2	0.25	2	0.25	0.125	0.5	0.25	0.063	1	0.5
Polymyxin B		4	0.5	1	0.25	1	0.125	0.125	0.5	0.25	0.063	0.5	0.125

Abbreviations: A, amide; E, ester.

Table S8. Fractional inhibitory concentration index (FICI) values of OligoG-colistin conjugates or colistin in combination with azithromycin dihydrate.

Tested Compound	<i>P. aeruginosa</i> MDR 301	<i>K. pneumoniae</i> KP05 506	<i>A. baumannii</i> 7789	<i>E. coli</i> NCTC 10418
OligoG-A-colistin + Azithromycin	1.35 (Additive)	3.15 (Indifferent)	3.40 (Indifferent)	0.64 (Additive)
OligoG-E-colistin + Azithromycin	1.45 (Additive)	2.43 (Indifferent)	2.53 (Indifferent)	0.46 (Synergy)
Colistin sulphate + Azithromycin	1.51 (Additive)	2.20 (Indifferent)	1.14 (Additive)	0.83 (Additive)