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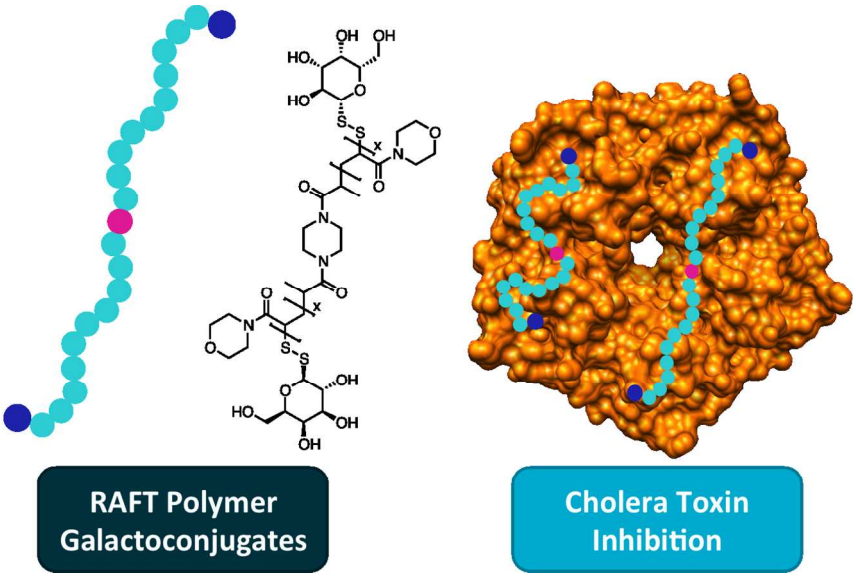
Complete List of Authors:	Leaver, David; La Trobe University, Chemistry Hughes, Andrew; La Trobe University, Chemistry Dawson, Raymond; DSTO, Postma, Almar; CSIRO, CMHT Malic, Nino; CSIRO, Materials Science and Engineering Polyzos, Anastasios; CSIRO, Materials Science and Engineering


RAFT Polymer Galactoconjugates
Cholera Toxin Inhibition

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David J. Leaver,[a,b] Andrew B. Hughes,[a] Raymond M. Dawson,[c] Almar Postma,[b] Nino Malic,[b] and Anastasios Polyzos,*[b]

We report a new strategy to develop low molecular weight (18-28 kDa) poly(*N*-acryloylmorpholine) (PNAM) polymers as bivalent inhibitors of cholera toxin (CT) using Reversible Addition-Fragmentation chain Transfer (RAFT) technology. The inhibitory activity of the galactoconjugated polymers was examined (ELISA) and the series displayed moderate inhibitory activity (millimolar IC_{50}).

Cholera is a severe diarrheal disease, caused by the Gram-negative bacterium *Vibrio cholerae*.[1] Cholera is also often linked with high infective and mortality rates and it has been estimated that approximately 120,000 deaths occur annually from cholera.[2,3] CT belongs to the AB_x class of toxins[4,5] and is comprised of a catalytic A-subunit and five identical B subunits that unite to form a pentamer that specifically binds to the cell surface receptor, ganglioside G_{M1}; a glycosphingolipid comprising an oligosaccharide head-group. Crystallographic studies have determined the mechanism for recognition of G_{M1} by the pentameric CTB-subunits[5] in which the terminal galactose of the G_{M1} oligosaccharide binds to a shallow binding epitope on each B subunit.

Appropriate galactose derivatives have the potential to disrupt the CTB:G_{M1} interaction and enable prophylactic or therapeutic drugs to be designed that can be used to combat the symptoms associated with *V. cholerae* infection. One such approach is a bivalent inhibitor design, in which two galactose units are terminally tethered to a linear linker.[6,7] The galactose units simultaneously bind to the CTB pentamer to overcome the low intrinsic affinity for single galactose-protein binding.

In our previous work we reported on the synthesis and biological activity of bivalent inhibitors comprising 1,2,3-triazole linked galactopyranosides against CT.[6] These galactotriazoles were designed to promote pendant galactose group binding to adjacent binding epitopes on the CT_B subunit (35 Å) based on the extended conformation of the polyethylene glycol (PEG) linking units. However, effective linker lengths are considerably shorter than corresponding extended lengths and it was anticipated that extension of the bivalent linkers based on effective dimensions would result in affinity gains against CT. The extension of polyethylene glycol (PEG) derivatised linkers *via* the use of squarate or guanidine groups is challenging owing to the synthetic difficulty of accessing extended derivatives through chain elongation that is characterized by repeated protecting/deprotecting steps and low solubilities of the synthetic intermediates.[8-12]

In order to address these challenges we turned our attention to Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization to enable the facile preparation of water soluble linkers of precise lengths that could be galacto-conjugated upon post-polymerization reactions.[13-17] The advantages of RAFT polymerization relative to other radical processes, include the ability to obtain polymers that are well-defined (low polydispersities) with predetermined molecular weights, 'living' polymerization characteristics and are amenable to conventional free-radical polymerization conditions. Furthermore the technique exhibits a tolerance to a wide range of functional groups and

flexibility to enable complex architectures to be generated, with requisite polarity to permit solubility in aqueous solutions.[15,18,19]

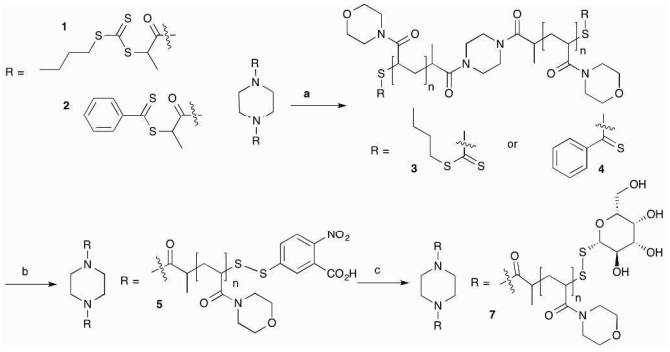
We now report the synthesis and biological evaluation of bivalent galactoconjugated RAFT polymers, which comprise the following design components: 1. Piperazine core, 2. Poly-N-Acryloylmorpholine PNAM linker, 3. Terminal thio-galactose moiety. N-Acryloylmorpholine (NAM) was chosen as the preferred monomer as poly(N-acryloylmorpholine) (PNAM) is water-soluble and known to have non-immunogenic properties similar to that of PEG.[18,20]

The optimal length of the PNAM linker was estimated by using effective linker lengths (Table 1).[5,12] It was determined that a length of approximately 146 NAM units would enable the bivalent ligands to reach the non-adjacent binding sites of CT (56 Å). As control measurements, PNAM RAFT polymers containing slightly shorter and longer effective linker lengths were also prepared.

Table 1 Properties of galacto-conjugated PNAM RAFT polymers

Polymers	Theor M_n	NMR M_n	GPC M_n	PD	Effective linker length (Å)
NAM 126 S	18500	18300	11700	1.08	50.7
NAM 130 S	19000	18600	12300	1.14	51.7
NAM 166 S	24100	22500	15300	1.09	60.3

The forward synthesis of the trithiocarbonate terminated RAFT polymers (**3**) is shown in Scheme 1. Trithiocarbonate RAFT agent **1** was employed to control the polymerisation process of NAM with the use of AIBN as the initiator.[20] This approach was utilised to obtain the target polymer NAM 130 S with an effective linker length of 51.7 Å (predicted optimal length 146 NAM units). The **NAM 126** and **NAM 166** polymers possessed slightly shorter (50.7 Å) and longer (60.3 Å) effective linker lengths respectively. The length of the PNAM backbone was controlled by varying the mole equivalents of NAM with respect to the RAFT agent **1**.



Scheme 1. The forward synthesis of RAFT polymers. *Reagents and conditions:* **a** *N*-acryloylmorpholine, AIBN, dioxane, 60ºC; **b** 5,5'-dithiobis-(2-nitrobenzoic acid), *n*-butylamine, water; **c** 1-thio-β-D-galactopyranoside, phosphate buffer pH 8.

The molecular weight of the dithioester PNAM RAFT polymers was controlled by limiting the reaction time (with a constant ratio of NAM to dithioester RAFT agent (**2**)).

The removal of the RAFT end-groups was necessary for glycosylation of the RAFT polymers. It was anticipated that disulfide exchange reactions[18,19,21-24] would suppress the formation of undesired disulfide formation and by using mixed disulfides an appropriate thio-galactose derivative would be conjugated to the RAFT polymers.

Both 2,2'-dithiodipyridine and Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid)) are known to form stable activated mixed disulfides with thiol terminated RAFT polymers, which can facilitate conjugation to various biomolecules such as oligonucleotides, carbohydrates, and peptides.[17-19,21-25] Ellman's reagent was chosen as the coupling agent to form the mixed disulfide (**5**) (Scheme 1) with the thiol terminated RAFT polymers that were generated from dithioester or trithiocarbonate RAFT polymers (**3** or **4**) upon *in situ* aminolysis. The mixed disulfides comprised of Ellman's reagent are susceptible to nucleophilic attack by the thiogalactoside owing to the enhanced electrophilicity promoted by the electron-withdrawing nature of the *para*-substituted nitro group. ^{1}H NMR (Fig. S3, Supporting Information) confirmed the attachment of Ellman's reagent to the PNAM RAFT polymers with characteristic aromatic peaks at 7.5 and 7.7 ppm.

The 2-nitro-5-thiobenzoate terminated RAFT polymers (**NAM 126 E – NAM 166 E**) were then subjected to disulfide exchange (Scheme 1) with 1-thio-β-D-galactopyranoside[26] to produce the glycosidated derivatives (**7**). Complete disulfide exchange of Ellman's group with 1-thio-β-D-galactopyranoside was achieved at pH 8 in a phosphate buffered solution.[22] This procedure promoted complete disulfide exchange of the Ellman's capped RAFT polymers (**NAM 126 E - NAM 166 E**) with 1-thio-β-D-galactopyranoside to form the desired galacto-conjugated RAFT polymers (**NAM 126 S – NAM 166 S**). Complete conversion for the disulfide exchange reaction was confirmed by the absence of the aromatic signals in the ^{1}H NMR spectrum (Fig. S3(iii), Supporting Information) associated with Ellman's group and the appearance of a signal peak at 4.3 ppm, which is characteristic of the anomeric hydrogen of the conjugated 1-thio-β-D-galactopyranoside. The estimated isolated yield using this method is > 90% for the polymer series. The use of mixed disulfides to install the pendant galactose units to the NAM RAFT polymers proved to be an effective glycosidation method, which was not prone to the formation of high molecular weight disulfides.

The inhibitory activity of the bivalent RAFT derivatives against the binding of 10 ng mL^{-1} CT to its cell surface receptor (G_{M1}) was determined as described previously.[27] The results from the biological analysis are shown in Table 2. **NAM 126 S** and **NAM 166 S** were assayed against CT in addition to **NAM 130 S** to examine the influence of the PNAM linker length on CT binding inhibiton. It is evident from the data in **Table 2** that **NAM 130 S** exhibits the greatest degree of affinity for CT (IC_{50} = 1.34 mM) from the three polymers assayed. The slightly longer 1-thio-β-D-galactopyranoside capped RAFT polymer **NAM 166 S** (IC_{50} = 2.32 mM) displayed improved inhibitory activity in comparison with the shorter analogue **NAM 126 S** (IC_{50} = 2.51 mM) and both were approximately half as potent as **NAM 130 S**. These results are

consistent with what is expected when the effective linker lengths are either shorter or longer than the optimal length. [9,12]

As a control measurement, the single galactose unit, 1-thio-β-D-galactopyranoside was screened against CT to establish whether the moderate activity observed for the RAFT polymers was due to a weak multivalent effect. If the 1-thio-β-D-galactopyranoside capped RAFT polymers were binding in a monovalent fashion to CT then the activity of these polymers would be expected to be close to the activity observed for 1-thio-β-D-galactopyranoside. **NAM 130 S** was observed to be 119 times more potent than 1-thio-β-D-galactopyranoside (Table 2), which suggests a multivalent effect is responsible for the enhancement of affinity of **NAM 130 S**. This multivalent effect is also evidenced in the other RAFT polymers (**NAM 126 S** and **NAM 166 S**) which also displayed improved potency over 1-thio-β-D-galactopyranoside.

Table 2. Data for inhibition of binding of cholera toxin to its receptor. [a]

Entry	Inhibitor	% Inhib. [b]	IC50 (mM)	Mw (theor.)
1	**NAM 126 S**	-	2.51 ± 0.21	18500
2	**NAM 130 S**	-	1.34 ± 0.050	19000
3	**NAM 166 S**	-	2.32 ± 0.19	24100
4	**NAM 126**	12 ± 2	-	18300
5	**NAM 130**	15 ± 2	-	18900
6	**NAM 166**	10 ± 3	-	24000
7	1-thio-β-D-galactopyranoside		159 ± 8	-

[a] Data are quoted as mean (arithmetic for % inhibition, geometric for IC50) ± standard error of the mean from 3 experiments. [b]. The concentration for the control polymers NAM 126, NAM 130 and NAM 166 is 15.44 mM, 13.80 mM and 11.78 mM, respectively.

As further control measurements, the RAFT polymers **NAM 126**, **NAM 130** and **NAM 166** without pendant galactosides were assayed against CT (Table 2) to determine if the enhanced affinity observed for the 1-thio-β-D-galactopyranoside RAFT polymers was a consequence of non-specific binding of the NAM polymer backbone. It can be observed from Table 2 that the underivatised RAFT polymers **NAM 126**, **NAM 130** and **NAM 166** are essentially inactive (10-15% inhibition) against CT, which indicates that non-specific binding of the NAM polymer backbone is not responsible for the improvement in CT affinity. This confirms that the multivalent effect has been observed for the 1-thio-β-D-galactopyranoside RAFT polymers.

Although direct comparisons of the IC_{50} values of the 1-thio-β-D-galactopyranoside PNAM RAFT polymers to related polymeric inhibitors should be made cautiously owing to the variation of results arising from different assay methods, a cursory collation of affinity data enables a qualitative analysis of structure-activity relationships of such systems. As such, **NAM 130 S** is slightly less potent against CT in comparison with other polymers such as a glycopeptide (IC_{50} = 0.25 mM) that was derivatised with *N*-linked galactotriazoles.[28] **NAM 130 S** is of comparable potency to shorter glycopeptides with random coil conformations.[29] In direct comparison with another study, **NAM 130 S** is more active than densely glycosylated poly(L-glutamic acid) glycopolymers.[30] The novel 1-thio-β-D-galactopyranoside capped RAFT polymers reported herein have reasonable affinity for CT, which is likely to be further enhanced upon future optimisations of conjugating potent small molecules to analogous polymeric scaffolds.

This work has established a new strategy to the expedient and facile synthesis of bivalent CT inhibitors using RAFT polymerization of NAM with either trithiocarbonate or dithioester RAFT agents. The use of RAFT polymerization enabled superior control of the linker lengths to be attained. Bivalent CT inhibitors were synthesised *via* disulfide exchange reactions, which successfully installed the pendant galactose groups and prevented the formation of undesired disulfide products. **NAM 130 S** was the most potent CT inhibitor reported herein with an IC_{50} of 1.34 mM. It is envisioned that upon optimisation of the ligand conjugated to the polymer backbone that dendrimitic structures could be obtained that might be able to be used as a cholera prophylactic/therapeutic drug and we are presently undertaking this work in our laboratories.

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Notes and references

[a] *Department of Chemistry, La Trobe University, Melbourne, Victoria, 3086, Australia.*
[b] *CSIRO Materials Science and Engineering, Bayview Avenue, Clayton South, Victoria 3169, Australia. E-mail: tash.polyzos@csiro.au*
[c] *DSTO Melbourne, Defence Science and Technology Organisation, P.O. Box 4331, Melbourne, Victoria 3001, Australia.*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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