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Strong inhibition of cholera toxin B subunit by affordable, polymer-based multivalent inhibitors

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ABSTRACT: Cholera is a potentially fatal bacterial infection that affects a large number of people in the developing countries. It is caused by the cholera toxin (CT), an AB_5 toxin secreted by *Vibrio cholera*. The toxin comprises of a toxic A-subunit and a pentameric B-subunit that binds to the intestinal cell surface. Several monovalent and multivalent inhibitors of the toxin have been synthesized but are too complicated and expensive for practical use in developing countries. Meta-nitrophenyl α-galactoside (MNPG) is a known promising ligand for CT and here mono- and multivalent compounds based on MNPG were synthesized. We present the synthesis of MNPG in greatly improved yields and its use while linked to a multivalent scaffold. We used economical polymers as multivalent scaffolds, namely polyacrylamide, dextran and hyperbranched polyglycerols (hPGs). Copper catalyzed alkyne azide cycloaddition reaction (CuAAC) produced the inhibitors that were tested in an ELISA-type assay and an intestinal organoid swelling inhibition assay. The inhibitory properties varied widely depending on the type of polymer and the most potent conjugates showed IC_{50} values in the nanomolar range.

Cholera is a disease that affects a large number of people in the developing countries due to limited access to safe drinking water and adequate sanitation. It is characterized by watery diarrhea which can rapidly be fatal when left untreated.[1] The annual burden of cholera has been estimated at 1.3 to 4.0 million cases and 21,000 to 143,000 deaths worldwide.[2] The recent cholera outbreak in Yemen has been called the world's worst cholera epidemic and has claimed 2200 lives in the year 2017 with more than a million suspected cases.[3] Treatment for cholera involves the use of oral rehydration therapy and antibiotics. There are three oral cholera vaccines: Dukoral®, Shanchol™, and Euvichol-Plus®/Euvichol® which are WHO pre-qualified and widely used but not very effective for children under five years of age.[4] Vaxchora® has recently been approved by the USFDA as a vaccine for adults who travel to an area of active cholera transmission.[5]

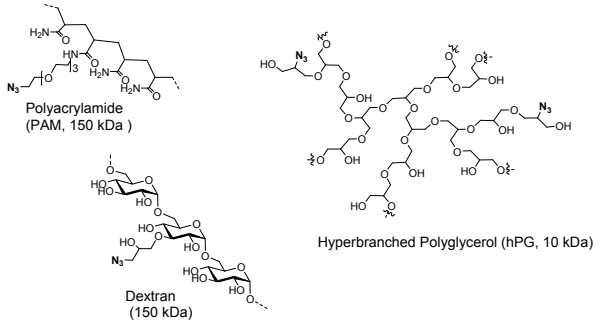


Figure 1. Polymers used for the multivalent presentation of Cholera toxin ligands

Cholera is caused by the cholera toxin (CT) which is an AB_5 toxin secreted by the *Vibrio cholera* bacterium. The core of the toxin consists of the A subunit which is responsible for the toxicity, surrounded by the pentameric B subunit. The B subunit enables the attachment of the toxin to GM1 ganglioside molecules on the intestinal cell surface which leads to endocytosis where the A subunit catalyses ADP ribosylation of G-proteins leading to increase adenylate cyclase activity.[6] This leads to increased intracellular cAMP which results in a chloride outflow leading to water secretion and diarrhea.[7] Therefore, preventing the entry of the toxin into the cell by blocking its attachment to the GM1 ganglioside is thought to be a good target for development of prophylactic drugs.[8,9] The high-affinity binding interaction of GM1-CTB (K_d = 43 nM) has been demonstrated using Isothermal titration calorimetry (ITC).[10] A novel secondary binding site has been recently identified that binds to fucosylated molecules including fucosylated blood group antigens although the binding is weaker with a K_d of 1.4 mM for the LeY-CTB binding.[11,12] Another closely related AB_5 toxin is heat-labile enterotoxin (LT1-B) that shares 80% sequence homology with CTB and causes traveler's diarrhea.[9]

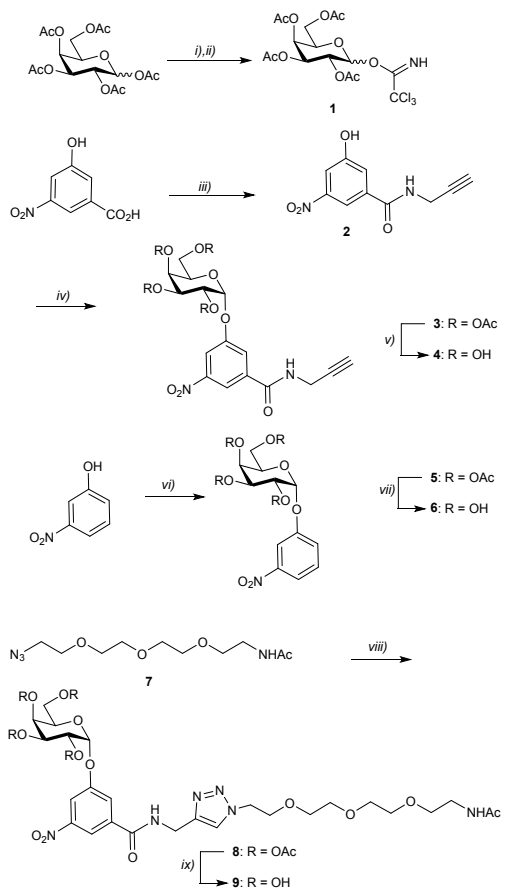
GM1 mimics, as monovalent inhibitors of CTB[13,14] and divalent[15,16,17,18] inhibitors based on different scaffolds have been developed. Multivalent inhibitors based on different scaffolds such as dendrimers,[19] calix[5]arene,[15] polymers,[20,21] and glycopeptides[22] have been synthesized. The most potent inhibitors are multivalent in nature and based on the GM1 oligosaccharide (GM1os).[23,24,25,26,27] These systems are all very

potent and inhibit in the nano- and picomolar range. Unfortunately, the structural complexity of GM1 and therefore the costs involved in preparing it does not make it an ideal candidate for drug development. The oligosaccharide of GM1 is very costly with hundreds of dollars for milligram amounts. Affordability is key, especially considering that the therapeutic needs to be repeatedly administered due to the natural flow of the intestinal tract, in order to maintain protection during epidemics. Thus, there is an urgent need for potent and economical inhibitors of CTB. To this end, researchers have conjugated galactose ligands to polymers and evaluated their potencies.[28] Among the best examples are Poly(L-glutamic)acid that was used and inhibitors reached IC_{50}'s in the 40-50 micromolar range, a 600-fold enhancement over monovalent galactose.[21] Similar results were reported with polyacrylamide linked galactoside, but a comparison with monovalent galactosides was not made.[20] Tran *et al.*[29] described a compound search of galactoside derivatives that were screened while linked to polyacrylamide, and yielded potent conjugates, although the monovalent compound itself was of similar potency to MNPG used in our study (*vide infra*). Jones *et al*.[30] used a similar approach. Based on these results we aimed to make a polymeric CTB antagonist of sufficient potency that is readily made and consists of affordable components. Within these constraints, this should include an optimal monovalent ligand combined with an optimal polymeric pharmaceutically benign backbone. The word sufficient potency is based on the concentration of cholera toxin present during cholera attacks in the intestinal tract which may reach close to 1 μM of B-subunits.[31,8] Submicromolar Kd's are therefore sufficient while picomolar inhibitors provide little advantage, as they still need to be present in micromolar inhibitor concentration to neutralize all the toxin.

Meta-nitrophenyl α-galactoside (MNPG) was the main candidate for our monovalent compound, which was discovered by Minke *et al.* almost two decades ago as a promising monovalent inhibitor in a screening assay where it exhibited a 100-fold increase in potency over D-galactose.[32] Its potency increase over galactose was thought to be entropy driven. This was explained by a new hydrogen bond formed with the nitro group liberating a conserved water molecule, in addition to increasing the surface of the ligand that can interact with the protein.[32] Further optimization of MNPG did not yield satisfactory results.[33,34] A multivalent version was potent when linked to a pentacyclen core (IC_{50} 6 nM) however, the conjugate was not stable,[35] and the result of a long and expensive synthesis. A major barrier was also the synthesis of MNPG and even more so for a version amenable to conjugation. Single digit yields combined with the need for an enzyme to separate the anomeric mixtures precluded the use of MNPG for applications.[33]

In the present paper, we focused on the synthesis of MNPG and a version suitable for conjugation and subsequently presenting it in a polymeric multivalent system. For this purpose, we have used the linear polyacrylamide and dextran with periodic branching, typically less than 10%, readily available and economical (Figure 1). The third polymer was considerably more branched: a hyperbranched polyglycerol (hPG) and known to be biocompatible and the 10 kDa variant is known to have a more globular nanoparticle shape with a diameter of 5-6 nm, substituted or not.[36,37,38,39,40] All three polymers were used as a scaffold by introducing azido functions and linking MNPG by Copper catalyzed alkyne azide cycloaddition (CuAAC) conjugation. The resulting glycopolymers were potent cholera toxin inhibitors, but the activity varied widely with the type of polymeric scaffold. Compounds were evaluated using an ELISA assay, but some compounds were also tested in our recently reported organoid assay.[41] Traditionally, rabbit ileal loop assay has been used to study enterotoxins including CT. However, the assay is not widely used to evaluate CT inhibitors as it is extremely stressful to the animals, time-consuming and not easy to standardize. The organoid assay overcomes these issues and could serve as a precursor to the animal model studies.

Scheme 1. Synthesis of MNPG and derivatives[a]



[a]Reagents and conditions: (i) $BnNH_2$, DMF, r.t., quantitative, (ii) Trichloroacetonitrile, K_2CO_3, CH_2Cl_2, 96%, (iii) Propargylamine, EDCI, DMAP,76%, (iv) **1**, TfOH, -35°C-r.t. 40-44%, (v) NaOH,MeOH, 85%, (vi) **1**, TfOH, -35°C-r.t. 58%, (vii) NaOH,MeOH, 83%, (viii) **3**, $CuSO_4$, Na. ascorbate, microwave, 75%, (ix) NaOH,MeOH, 73%.

RESULTS

Synthesis. A major limitation of the use of MNPG as a ligand suitable for conjugation, was its difficult, poorly described and extremely low yielding synthesis.[33] In our hands eventually an imidate-based, TfOH catalyzed glycosylation proved to yield the desired α isomer with reasonable yields. 3-hydroxy-5-

nitrobenzoic acid was used as the starting point for the synthesis in which the acidic group was converted to a propargyl amide **2** (Scheme 1). Galactose imidate **1**[42] was used as the glycosyl donor and a glycosylation reaction was performed using triflic acid as the promoter wherein phenol (**2**), obtained from 3-hydroxy-5-nitrobenzoic acid, served as the acceptor. The glycosylation product (**3**) was obtained in 40-44%. It was then deprotected to give the MNPG ligand **4** that was used for the 'click' reaction with the different polymeric scaffolds. We have synthesized unsubstituted MNPG using 3-nitrophenol as the glycosyl acceptor and galactose imidate as the donor in the triflic acid promoted glycosylation to give the α-isomer **5** in 58% yield, which was deprotected to give MNPG **(6)**.

Besides compounds **4** and **6**, a third MNPG-based and more soluble monovalent compound was synthesized using 11-azido-3,6,9-trioxundecan-1-amine by first converting the amine to amide **7** and then conjugating it to **3** to give **8** which was deprotected to give **9**. 11-azido-3,6,9-trioxundecan-1-amine was also used for the conjugation of an azide moiety to polyacrylamide (Mn: 150 kDa) and dextran (Mn: 150 kDa) according to reported procedures[43,29] to give polymers **10** and **11** respectively (scheme 2). The incorporation of the azide group was confirmed by the appearance of the azide stretching peak at 2110 cm^{-1} in the infrared spectroscopy (IR) spectra. The molecular weights and the percent azide functionalization of **10** and **11** (1.7% and 0.6% respectively) were calculated by integrating relevant peaks in the proton NMR spectra.[43] A shorter azide containing appendage was also used to functionalize dextran.[44] To this end 1-azido-2,3-epoxypropane was used which was synthesized in two steps from epichlorohydrin (see supporting information). Polymer **12** was obtained with a 6% azide functionalization, as determined by proton NMR and was conjugated to the MNPG ligand **4** and prop-2-ynylβ-galactoside using CuAAC to give **15** and **17** respectively. The complete disappearance of the azide stretching peak in each of the reactions confirmed that all of the polymer azide was consumed (see S.I.). hPG (Mn: 10,000) with a 10% azide functionalization was prepared as before[40] and used for conjugation to **4** to give glycopolymer **18**.

Cholera Toxin Inhibition. The synthesized compounds were tested for their ability to inhibit the cholera toxin B-subunit by making use of the well-established GM1-based ELISA assay. Galactose was used as a reference inhibitor and was the least potent molecule with an IC_{50} of 111 mM (Table 1). Monovalent inhibitors MNPG (**6**) and its derivative **9** showed inhibition in the low millimolar range. MNPG emerged as the best monovalent ligand with a 58-fold potency increase over galactose (IC_{50} 1.9 mM) which is comparable to the reported enhancement values. Compound **9** showed 27-fold potency gain over galactose (IC_{50}: 4 mM). All of the multivalent compounds showed at least inhibition in the low micromoler range. Dextran-based Compound **15** and hPG- based compound **18** emerged as the most potent with comparable IC_{50} values in the nanomolar range (390 and 530 nM respectively). Dextran-MNPG conjugate **15** was more potent than the dextran-galactose conjugate **17** despite the same number of azides on the dextran thereby confirming the inhibitory potential of MNPG. Polyacrylamide-MNPG conjugate **13** and dextran conjugate **14** also showed significant inhibition (IC_{50}'s 5.6 μM and 8.4 μM).

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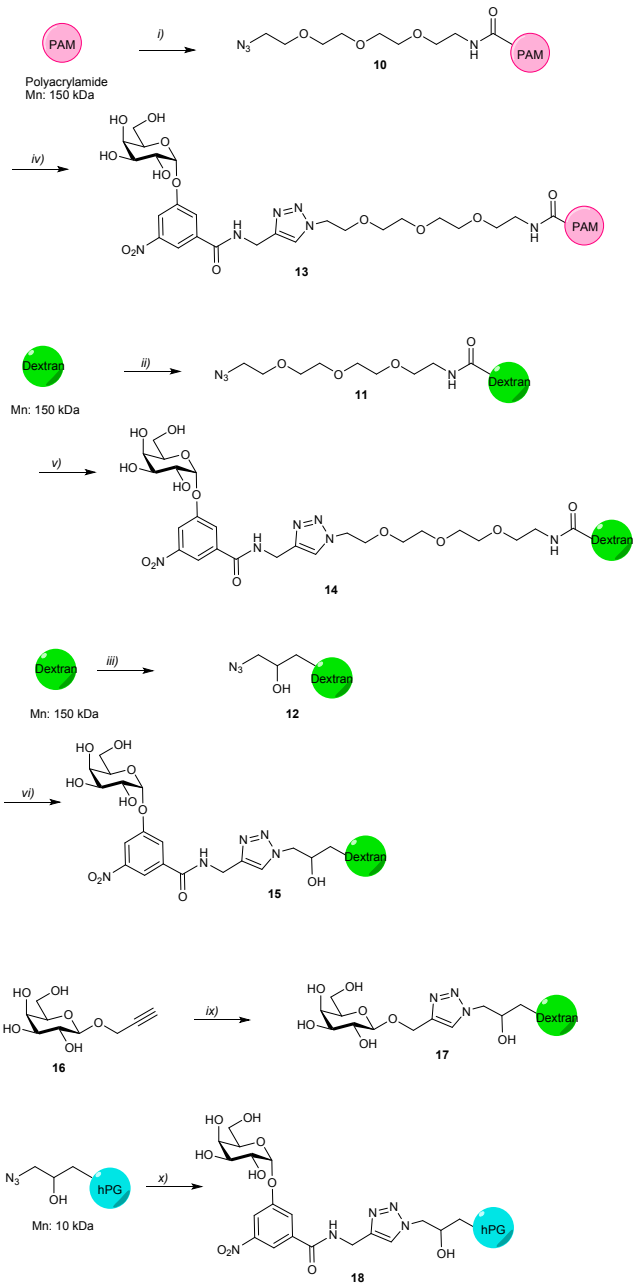
Scheme 2. Synthesis of MNPG- and galactose-based polymers[a]



Scheme 2: i) 11-Azido-3,6,9-trioxaundecan-1-amine, 80°C, 60 min.,95%, ii) 11-Azido-3,6,9-trioxaundecan-1-amine, CDI, DMAP, DMSO, r.t., 48h, iii) a) iPrOH, AcOH, NaN_3, Epichlorohydrin, 5M NaOH, b) 5M NaOH, iv) **4,** $CuSO_4$, Na. ascorbate,100°C, 78%, v) **4,** $CuSO_4$, Na. ascorbate,100°C, 83%, vi) **4,** $CuSO_4$, Na. ascorbate,100°C, 51-58%, ix) **12**, $CuSO_4$, Na. ascorbate,100°C, 35%, x) **4**, $CuSO_4$, Na. ascorbate,100°C, 62%.

Besides the ELISA assay, the best multivalent glycopolymers **(13,15,18)** along with all the monovalent compounds were evaluated for their inhibitory potential in our recently reported organoid assay.[41] To apply the organoid assay, intestinal organoids[45] were stimulated dose dependently with cholera toxin to select a non-saturating concentration for inhibitor testing while retaining maximal assay sensitivity (see S.I.). 3 μg/mL of cholera toxin was required to induce sufficient swelling of these patient derived organoids which is 30 times higher than was needed for previously used organoids derived from a different patient.[41] We next assessed dose-dependent inhibition of cholera toxin-mediated swelling of the inhibitors for binding cholera toxin B subunit. Galactose was measured as a reference inhibitor. Organoids were stimulated with cholera toxin, with or without inhibitors. We found that all the polymer-based compounds were potent inhibitors of cholera toxin-induced swelling, with IC_{50}'s in the low micromolar range Table 2). hPG-based inhibitor **(18)** was the most potent (6.9 μM) with **13** and **15** showing comparable inhibition (IC_{50} 15 μM and 12 μM). Galactose was the least potent (IC_{50} 100 mM) with MNPG **6** and **9** inhibiting in the low millimolar concentration (IC_{50} 13 mM and 9.9 mM respectively).

DISCUSSION

We have synthesized and evaluated glycoconjugates as inhibitors of the cholera toxin. Our previous dendrimers containing GM1os were very potent, but not economically practical for application.[46,41] To put this into perspective, the GM1 sugar that was used for the synthesis currently (2018) costs ca. 400 USD per miligram whereas the polymers used by us only cost a fraction i.e. just a dollar or so per gram, which is also the case for 3-hydroxy-5-nitrobenzoic acid, while bulk prices are much lower. For the first time, we described a step-by-step synthesis of MNPG whereby it can be easily synthesised and purified in good quantities. The fact that only the α-isomer of MNPG and derivatives was obtained may be due to the anomeric effect providing enhanced stability for the α-isomer caused by the electron withdrawing nitrophenyl group. TfOH likely catalyzes the anomerization to the α-isomer even if the β-isomer was formed also. Similar observations were made previously.[47] We also developed a reaction strategy to incorporate it in a multivalent system by synthesizing compound **4**. Following this, we have successfully incorporated it into the polymeric scaffolds (i.e. polyacrylamide, dextran and hPG) of varying azide functionalization for the synthesis of the final multivalent compounds. The inhibitory potential of the synthesized compounds was evaluated using the GM1 ELISA protocol. Additionally, an intestinal organoid swelling inhibition assay was performed which is a more biorelevant *in vitro* assay to confirm the

Table 1. Results of inhibition by multivalent carbohydrates in CTB-HRP ELISA assay.[a]

entry	construct	ligand	Valency (% functionalization of polymer)	IC_{50} (μM)	rel.pot.[b]	rel. pot. per sugar[c]	IC_{50} (μg/mL)
1	galactose	gal	1	111,500 ±10,000	1	1	20,087
2	**6**	MNPG	1	1,907 ± 420	58	58	574
3	**9**	MNPG	1	4095 ± 230	27	27	2,628
4	**17**	gal	55 (6%)	6.6 ±1	16,870	304	1,108
5	**13**	MNPG	36 (1.7%)	5.6 ±0.5	731	20	961
6	**15**	MNPG	55 (6%)	0.39 ±1	10,500	191	69
7	**14**	MNPG	6 (0.6%)	8.4 ±1	488	81	1,299
8	**18**	MNPG	13 (10%)	0.53 ±2	7,726	594	8

[a]determined in an ELISA-like assay with CTB_5-HRP (40 ng/mL) and wells coated with GM1, [b]relative to the potency of galactose for **6**, **9** and **17**, and to **9** for the rest, [c]relative potency divided by the valency.

Table 2. Results of inhibition of CT induced swelling of intestinal organoids by multivalent carbohydrates[a]

entry	construct	ligand	Valency (% functionalization of polymer)	IC_{50} (μM)	rel.pot.[b]	rel. pot. per sugar[c]	IC_{50} (μg/mL)
1	galactose	gal	1	100,000 ±10,000	1	1	18,015
2	**6**	MNPG	1	13,380 ± 1844	7	7	4269
3	**9**	MNPG	1	9,993 ± 1100	10	10	6413
4	**13**	MNPG	36 (1.7%)	15.5 ±5	644	18	2583
5	**15**	MNPG	55 (6%)	12 ±4	832	15	2121
6	**18**	MNPG	13 (10%)	6.9 ±2	1,448	111	110

[a]determined in an assay observing the swelling of intestinal organoids by CT as a function of inhibitor concentration, [b]relative to the potency of galactose for **6** and **9**, and to **9** for the rest, [c]relative potency divided by the valency.

inhibitory potential of the synthesized compounds. The most important measure for the potency enhancement imparted by the scaffold on which the ligands are presented is the potency per ligand. If a divalent ligand is twice as potent compared to the monovalent ligand it essentially provides no benefit, the relative potency per ligand is 1. The highest number observed here is 594-fold for **18**. This is a big number and clearly shows the large benefit of the hPG nanoparticle/polymer. The second best was galactose based-dextran conjugate **17** with a 304-fold potency enhancement over galactose. The same scaffold also yielded a high potency enhancement for MNPG linked to the same scaffold, but here the number was 191-fold per sugar. Interestingly, the most effective polymeric backbone seems to be the hPG especially when expressing its activity in terms of μg/mL of the whole polymeric construct. Its geometry is considered a nanoparticle with a ca. 5-6 nm diameter,[40] which matches the toxin diameter size (6-7 nm diameter) quite well. This is a feature that was recently shown to be favorable and of importance for strong inhibition, based on computational studies.[48] Our previous multivalent dendritic non-polymeric inhibitors, including a pentavalent one, were shown to aggregate the toxin by analytical ultracentrifuge measurements, which may have contributed to their potency.[49,50] One-on-one complexes have also been reported by DLS for a well-defined CTB_5-based inhibitor,[23] as well as 2:1 complexes for a decavalent system.[51] Based on this it is likely that the polymeric and nanoparticle inhibitors described here, that are of higher valency than our mentioned dendritic inhibitors, also bind to multiple toxins and induce aggregation that way. We here observed a distinct advantage of the nanoparticle hPG as the ligand scaffold over the linear polyacrylamide and the sporadically crosslinked dextran. A possible explanation is that a high number of ligands in a small area is beneficial as they can occupy several of the toxin binding sites simultaneously. The hPG also had the highest ligand density of 10 %, and for dextran the higher ligand density of **15** was beneficial in comparison to the 10 times lower functionalized of **14**. The hPG seemed to be the most potent due to a combination of the particle shape of suitable size and a relatively high functionalization. Even though the polyacrylamide and dextran backbones were previously shown[29] to be highly effective ligand scaffold, the hPG is clearly superior. This is particularly clear when expressing the potency in terms of μg/mL, where the weight of the polymer and the ligand density also play a role.

The cholera toxin inhibition observed here is of sufficient practical potency, that should be able to neutralize the up to micromolar quantities of the toxin B-subunits present in an active infection by repeated administration. The polyacrylamide backbone was the least effective in our study, and is suspect with respect to toxicity.[52] The dextran polymeric backbone is biodegradable, which is considered an advantage for our application,[29] and has also used by others in the intestinal tract.[53] The hPG nanoparticles have been studied in detail for their behavior in biological systems and found to be non-toxic.[54]

CONCLUSION

We have prepared a new potent conjugate between MNPG and the pharmaceutically benign hPG nanoparticle platform. The new synthesis makes MNPG readily accessible and the conjugate showed good potency against the cholera toxin B-subunit in two assays, with potential as a prophylactic drug in cholera epidemics.

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Notes

The authors declare no conflict of interest.

SUPPORTING INFORMATION

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Experimental details, NMR , inhibition curves for ELISA assay, inhibition curves for organoid assay and IR spectra.

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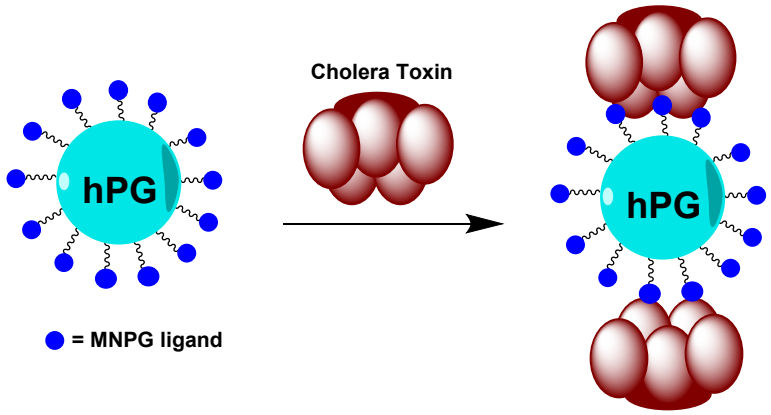
Graphical abstract



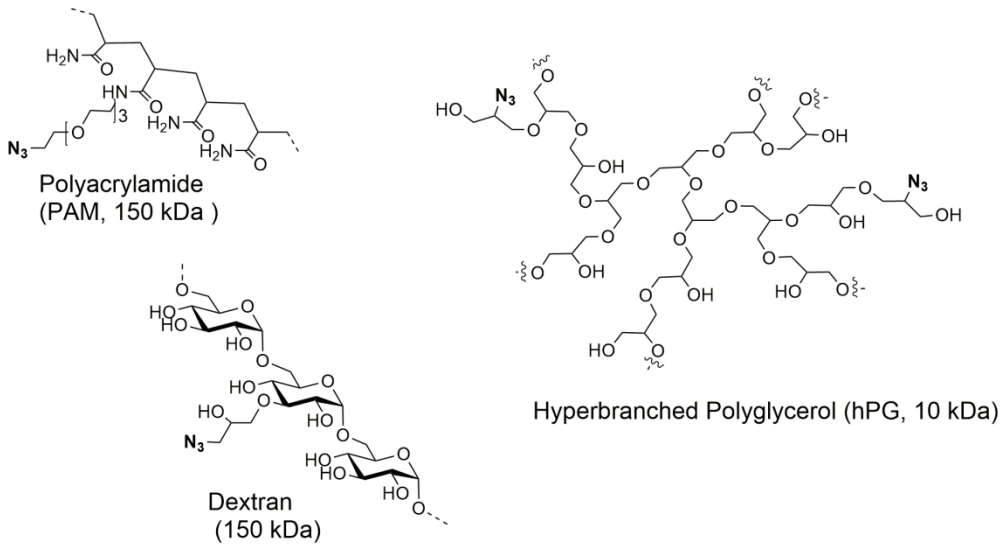


Figure 1. Polymers used for the multivalent presentation of Cholera toxin ligands

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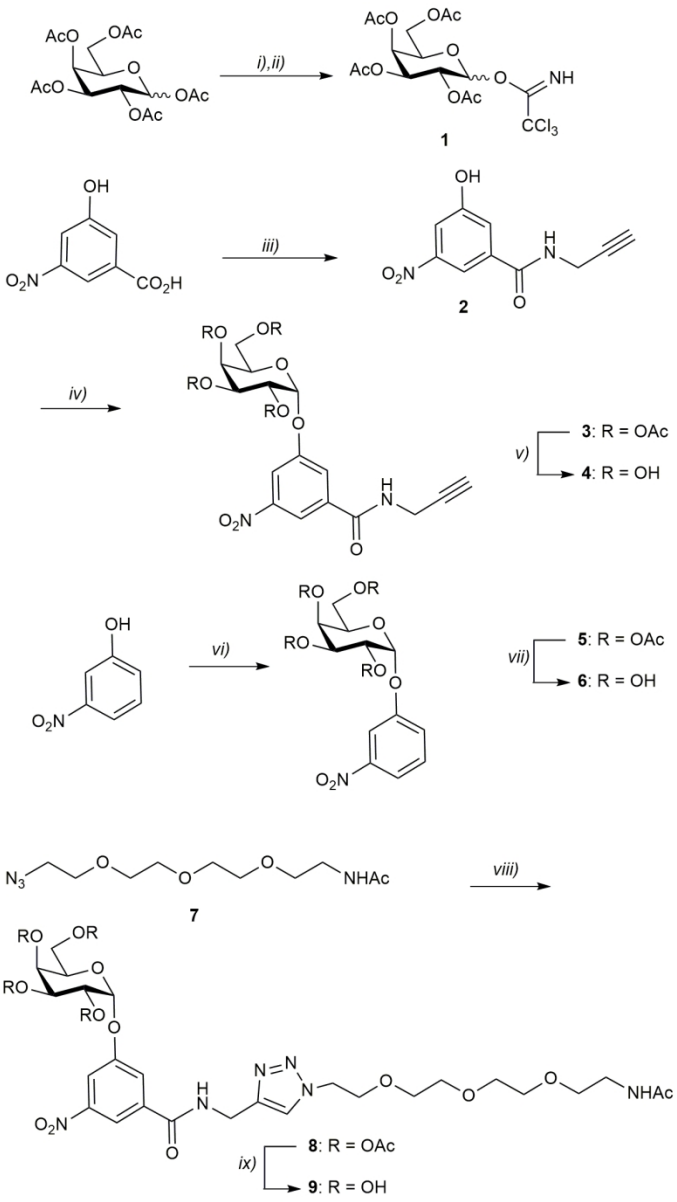
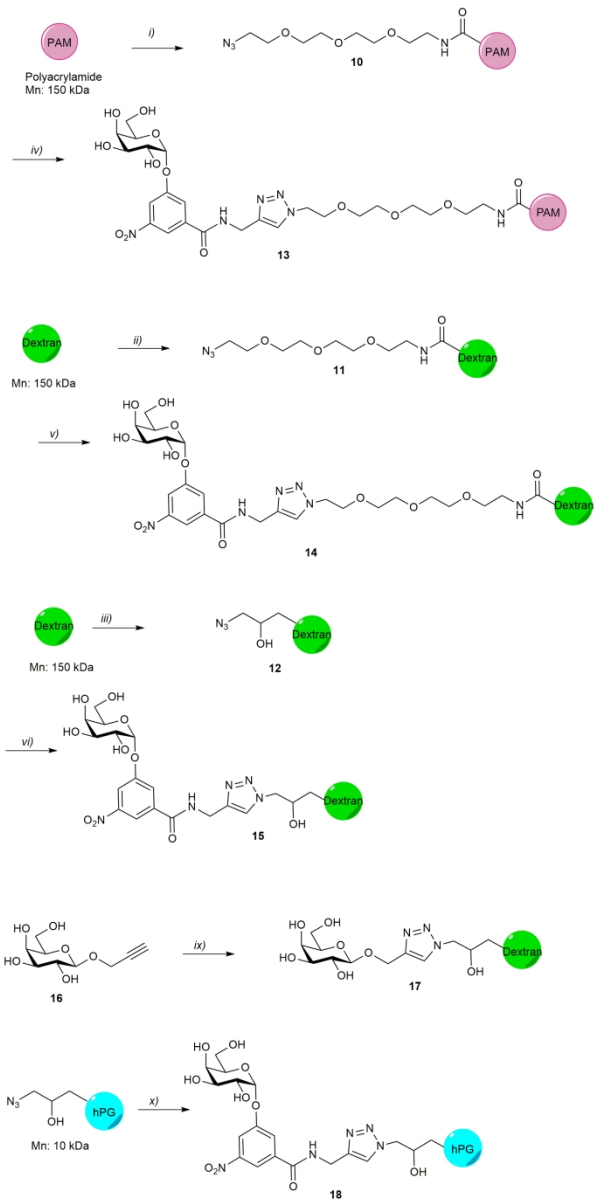


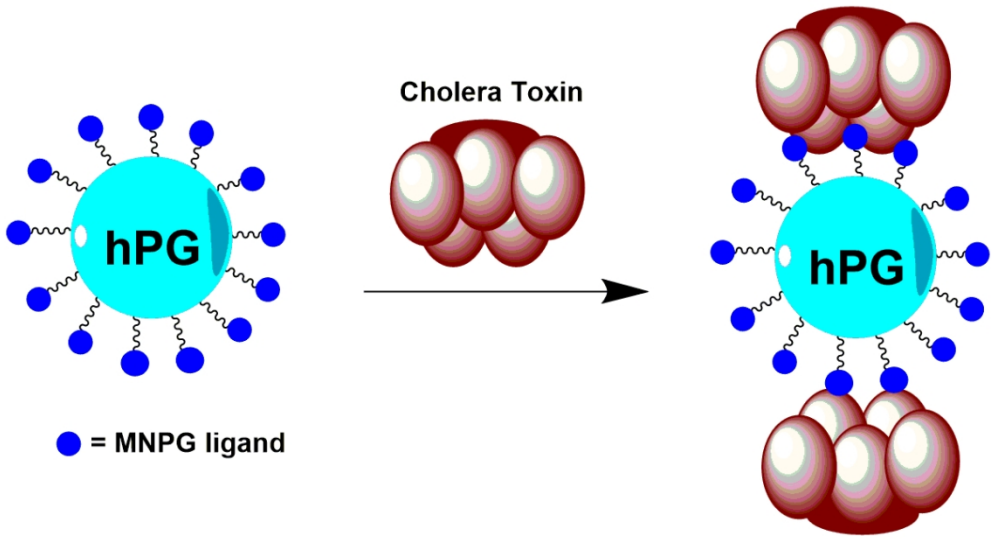
Scheme 1. Synthesis of MNPG and derivatives

108x191mm (300 x 300 DPI)



Scheme 2. Synthesis of MNPG- and galactose-based polymers

148x298mm (300 x 300 DPI)



Graphical Abstract

102x55mm (300 x 300 DPI)