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Synthesis and functionalization of virus-mimicking cationic block copolymers with pathogen-associated carbohydrates as potential vaccine adjuvants†

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We report the synthesis of a family of amphiphilic pentablock polymers with different cationic blocks and with controlled architectures as potential vaccine carriers for subunit vaccines. The temperature and pH-dependent micellization and gelation of these pentablock copolymers can provide a depot for sustained protein and gene delivery. The amphiphilic central triblock promotes cellular endocytosis, good gene delivery and has been used effectively as a vaccine adjuvant. The pentablock copolymer outer blocks condense DNA spontaneously as a result of electrostatic interactions for sustained combinational therapy. This family of polymers with different cationic groups was evaluated based on DNA complexation-ability and cytotoxicity to select promising candidates as DNA-based subunit vaccine adjuvants. Modification of other polymer systems with carbohydrates like mannose has been shown to enhance immunogenicity by activating pattern recognition receptors on antigen presenting cells and increasing uptake in these cells. Here, we report the synthesis of a virus-mimicking pentablock copolymer vaccine platform by successful functionalization of these polymers with mannose through an azide–alkyne Huisgen cycloaddition. The synthesis of a mannoside with the alkyne linker was achieved by a recently reported bismuth(v)-mediated activation of a thioglycoside that proved to leave the alkyne intact. The carbohydrate modification was shown not to interfere with the ability of these virus-mimicking block copolymers to complex DNA, thereby making this family of modified materials promising candidates for DNA-based vaccine delivery.

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Introduction

Block copolymers based on Pluronics have been used in a variety of applications, including sustained drug delivery, therapeutic cellular transfection and as novel vaccine adjuvants.[1–3] Pluronic F127 is an FDA-approved surfactant that exhibits temperature and pH-dependent gelation properties that make it an ideal carrier for hydrophobic drugs such as paclitaxel, aspirin and antibiotics.[4–8] Pluronic F127 has a central hydrophobic chain of polyoxypropylene and two outer hydrophilic chains of polyoxyethylene.[9] The hydrophobic interactions between the collapsed polyoxypropylene blocks result in the formation of micelles.[10] There are several advantageous properties of Pluronic that make it suitable for antigen delivery including cellular uptake through endocytosis and high gene expression.[3,4,9,11] There is also evidence that hydrophobic portions of synthetic polymers can initiate immune responses.[12,13]

Our research group has previously synthesized a family of cationic pentablock copolymers based on Pluronics for drug delivery.[14] The pentablock copolymers, which are synthesized using Pluronic macroinitiators and atom transfer radical polymerization (ATRP) of the outer cationic blocks, offer several critical advantages for DNA-based vaccine delivery. The amine groups on the pentablock copolymer outerblocks can form nanoscale complexes with DNA spontaneously as a result of electrostatic interactions.[15] Detailed studies with one of these pentablock copolymers with poly(2-diethylaminoethyl methacrylate) (PDEAEM) cationic blocks, have shown that the polymer micelles aggregate to form physical gels at high polymer concentrations. This enables the pentablock copolymers to serve as a unique multi-purpose platform to condense DNA, enable sustained gene delivery from the pentablock copolymer gels, and also provide combinational therapy of gene and drug co-delivery from the same platform.[10,16,17] However, the effect of different cationic blocks on DNA complexation ability as well as cytotoxicity of the copolymers has not been investigated. Herein

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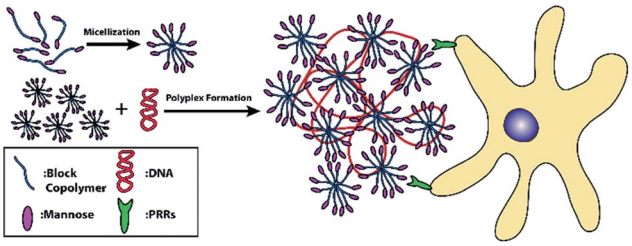


Scheme 1 Illustration of mannose functionalized pentablock copolymer condensing DNA and interacting with pattern recognition receptors (PRRs) on an antigen presenting cell.

we synthesize four pentablock copolymers based on Pluronic with end blocks of PDEAEM, poly(2-dimethylaminoethyl methacrylate), poly(2-diisopropylaminoethyl methacrylate) or poly-(*tert*-butylaminoethyl methacrylate) abbreviated PDMAEM, PDiPAEM and P*t*BAEM, respectively. These polymers with different cationic groups were then evaluated for suitability in a DNA-based vaccine formulation through DNA condensation and cellular cytotoxicity studies to select lead candidates.

To establish protective immunity, vaccines can enhance an innate immune response by activating antigen presenting cells (APCs).[18] Dendritic cells are a critical type of APCs that are present in all body tissues.[19] Immature dendritic cells can activate directly from pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs).[19] Carbohydrates; such as mannose, have been shown to act as PAMPs which can target carbohydrate-recognition receptors, specifically C-type lectin receptors abundantly expressed on dendritic cells.[20–22] Ligation of C-type lectin receptors with mannose structures displayed by pathogen glycoproteins leads to internalization of pathogens for antigen loading of major histocompatibility molecules.[21,23] Therefore, nano-scale adjuvants with attached carbohydrates can act as virus-mimicking particles such as mannose containing HIV-1 and measles virus, and increase dendritic cell transfection efficiencies through receptor mediated endocytosis and presentation of processed antigen to T cells.[22] In this work, we demonstrate the successful functionalization of this family of pentablock copolymer vaccine candidates with D-mannose through an azide–alkyne Huisgen click reaction.[24,25] Furthermore, gel electrophoresis studies show that the mannose functionalization of the copolymers does not affect the ability of the polymers to complex with DNA. A diagram showing the interaction of mannose functionalized pentablock copolymer DNA polyplexes interacting with PRRs on a dendritic cell can be found in Scheme 1.

Experimental section

Reagents

2-*N*,*N*-(Diisopropylamino)ethyl methacrylate (DiPAEM), *tert*-butylaminoethyl methacrylate (*t*BAEM) and inhibitor removal columns were purchased from Scientific Polymer Products Inc., Ontario, NY. *N*,*N*-(Dimethylamino)ethyl methacrylate (DMAEM), *N*,*N*-(diethylamino)ethyl methacrylate (DEAEM), Pluronic F127 (M_n = 12 600, 70% PEO), and D-mannose were purchased from Sigma-Aldrich, St. Louis, MO. Triphenyl bismuth was purchased from Strem Chemicals, Newburyport, MA. Slide-A-Lyzer® G2 Dialysis Cassettes (10 kDa M_w cut off) were purchased from Thermo Scientific, Rockford, IL. All other chemicals were purchased from Fisher Scientific, Pittsburgh, PA. The synthesis of *N*-propyl-pyridynyl methanimine (NPPM) from 1-propylamine and 2-pyridinecarbaldehyde has been previously reported.[14]

General experimental

Air- and moisture-sensitive reactions were carried out in oven-dried or flame-dried glassware, septum-capped under atmospheric pressure of argon. Commercially available compounds were used without further purification unless otherwise stated. The exact reaction conditions are given in the respective procedures. Air- and moisture-sensitive liquids and solutions were transferred *via* syringe or stainless steel cannula. All saccharides and the Bi(v) reagent were pre-dried by azeotropic removal of water using anhydrous toluene. Flash silica gel column chromatography was performed employing Silica Gel 60 Sorbent (40–63 μm, 230–400 mesh). Thin-layer chromatography (analytical) was performed using glass plates pre-coated to a depth of 0.25 mm detected under UV light and by spraying the plates with a 0.02 M solution of resorcinol in 20% ethanolic H_2SO_4 solution followed by heating.

Instrumentation

Proton (^{1}H) NMR, carbon (^{13}C) NMR, ^{1}H-^{13}C HSQC were recorded on a Bruker Avance III 600 MHz (or Varian VXR 300 MHz), 125 MHz instrument respectively using the residual signals from chloroform ($CDCl_3$), 7.26 ppm and 77.0 ppm as internal references for ^{1}H and ^{13}C chemical shifts (δ) respectively. Electrospray ionization high-resolution mass spectrometry (ESI-HRMS) was carried out on an Agilent 6540 QTOF. Optical rotations were measured at 20 °C using an automatic polarimeter AP300. Melting points were recorded in capillary tubes on a Digimelt SRS. IR spectroscopy was recorded by putting a thin film of the analyte on a salt plate on a Perkin-Elmer instrument.

Preparation of Pluronic macrointiator

Pluronic F127 (40.0 g, 3.20 mmol) was dissolved in tetrahydrofuran in a round-bottom flask over an ice bath. Triethylamine (20.0 mL, 143 mol) and 2-bromoisobutyryl bromide (3.00 mL, 24.2 mmol) were then added to the round-bottom flask. The reaction proceeded for 20 h up until the removal of solvent under reduced pressure and the precipitation of Pluronic macrointiator with *n*-heptane inside of a dry ice bath. The characterization of Pluronic macrointiator by ^{1}H NMR has been previously described.[14]

Synthesis & characterization of the pentablock copolymer family

The PDEAEM, PDMAEM, P*t*BAEM and PDiPAEM pentablock copolymers were synthesized *via* ATRP with the Pluronic macrointiator. The macrointiator (10.0 g, 0.780 mmol) and copper(I) bromide (0.240 g, 1.67 mmol) were dissolved in toluene inside

of an argon flushed single neck round bottom flask. The NPPM ligand (0.500 mL, 3.40 mmol) and respective monomer (2.50 mmol) were added and the reaction flask was degassed *via* three freeze–pump–thaw cycles. A small volume of inert gas was added and the reaction was stirred at 70 °C in an oil bath. After 20 h the reacted product was passed through a basic alumina column with a 1 : 1 dichloromethane–toluene solution. The eluate was concentrated under reduced pressure. The product was then precipitated using chilled *n*-heptane and left to dry in a vacuum oven maintained at 25 inches Hg vacuum (125 Torr).

DNA gel electrophoresis

To investigate the DNA condensation ability of polymers, the self-assembled pentablock copolymers were mixed with DNA in Hank's Balanced Salt Solution (HBSS) at different N/P ratios to form polyplexes. The polyplexes were briefly vortexed and incubated at room temperature for 20 min to ensure complexation. The samples were prepared in TriTrack sample buffer and a 15 μL volume was loaded onto a 0.70% agarose gel containing 0.1 μg mL^{-1} ethidium bromide. These polyplexes were run on an agarose gel with a constant of 1 μg of DNA per lane. The gel was run in Tris–acetate EDTA buffer at 75 V for approximately 2 h. All fluorescent images were captured on a UVP BioDoc-It® Imaging System with a 1.3 megapixel camera.

Cytotoxicity studies

The cytotoxicity of the different pentablock copolymers was tested using an *in vitro* CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega, Wisconsin, United States). EL4 C57BL/6 mice lymphoma cells were seeded on a 96 well plate with a density of 10 000 cells per well and incubated at 37 °C for 24 h in a humidified, 5% CO_2 atmosphere. Pentablock copolymers dissolved in HBSS Buffer were added at concentrations calculated from an NP ratio of 20 for 0.2 μg and 0.6 μg of DNA and incubated with the cells at 37 °C for an additional 24 h. A 15 μL volume of the Promega tetrazolium salt reagent was added to each well, the 96 well plate was incubated for an additional 4 h and then a 100 μL volume of the Promega solubilization solution was added to each well. After an additional 1 h of incubation, absorbance values were recorded with a microplate reader at 570 nm wavelength and a subtracted 690 nm reference wavelength. Corrected absorbance values were found by subtracting an average absorbance value of the 100% lysed positive control cells. The cell viability numbers were calculated by dividing the corrected absorbance value by an average of the non-treated negative control cells.

Functionalization of pentablock copolymer with azide linker

Pentablock copolymer (5.40 g) and sodium azide (0.243 g, 3.60 mmol) were dissolved in *N*,*N*-dimethylformamide and reacted at 50 °C for 24 h. The reacted product was precipitated in chilled *n*-heptane, collected using a Büchner funnel and left in a vacuum oven to dry.

Synthesis of alkynyl mannose analogue

***n*-Propyl-2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (2).** A 0.500 M solution of peracetylated donor **1** (ref. 26) (2.00 g, 5.77 mmol, 1.0 equiv.) and propanethiol (PrSH, 0.571 g, 7.50 mmol, 1.3 equiv.) were stirred for 0.5 h in anhydrous dichloromethane at 0 °C. Then, boron trifluoride diethyerate ($BF_3 \cdot OEt_2$, 1.23 g, 8.65 mmol, 1.5 equiv.) was added drop wise at 0 °C to the reaction mixture and then the reaction was left to stir at ambient temperature for 16 h. The reaction was quenched with excess triethylamine, diluted with CH_2Cl_2, filtered, and washed sequentially with 2 M aqueous HCl, saturated aqueous $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure at 40 °C; the resulting residue was purified by silica gel column chromatography with hexane:ethyl acetate (4 : 1) to yield **2** as a white solid (1.95 g, 4.78 mmol, 83%); R_f 0.48 (hexanes–ethyl acetate 3 : 1); $[\alpha]^D$ +73.3 cm^3 g^{-1} dm^{-1} (*c* 0.012 g cm^{-3}, $CHCl_3$; ^{1}H NMR (600 MHz, [D1] $CDCl_3$, 25 °C, TMS) δ = 5.32 (dd, *J* = 3.1, 1.6, 1H; H-3), 5.28 (m, 1H; H-2), 5.25 (d, *J* = 3.1, 1H; H-1α), 5.23 (m, 1H; H-4), 4.37 (ddd, *J* = 9.3, 5.4, 2.3, 1H; H-5), 4.28 (dd, *J* = 12.2, 5.5, 1H; H-6a), 4.07 (dd, *J* = 12.3, 2.4, 1H; H-6b), 2.66–2.47 (m, 2H; $SCH_2CH_2CH_3$), 2.15–1.94 (m, 12H; 4 × $CH_3C{=}O$), 1.69–1.57 (m, 2H; $SCH_2CH_2CH_3$), 0.97 (t, *J* = 7.3, 3H; $SCH_2CH_2CH_3$). ^{13}C NMR (151 MHz, [D1] $CDCl_3$, 25 °C, TMS) δ = 170.56, 169.96, 169.75, 169.72 (4C; C=O), 82.64 (1C; C-1), 71.21, 69.46, 68.91, 66.37 (4C; C-2,3,4,5) 62.45 (1C; C-6), 33.48 (1C; $SCH_2CH_2CH_3$), 22.80 (1C; $SCH_2CH_2CH_3$), 20.92, 20.70, 20.69, 20.62 (4C; 4 × $CH_3C{=}O$), 13.35 (1C; $SCH_2CH_2CH_3$), HRMS (ESI-QTOF) *m*/*z* calc. for $C_{17}H_{26}O_9SNa^+$ 429.119 found 429.126.

2-Propynyl-2,3,4,6-tetra-*O*-acetyl-1-*O*-α-D-mannopyranoside (3). A 0.50 M solution of thioglycoside donor **2** (0.250 g, 0.615 mmol, 1.0 equiv.) and propargyl alcohol (0.035 g, 0.615 mmol, 1.0 equiv.) were stirred together in anhydrous dichloromethane (CH_2Cl_2) for 0.5 h in a flask filled with Ar at ambient temperature. A solution of $Ph_3Bi(OTf)_2$ (ref. 27) (0.317 g, 0.430 mmol, 0.700 equiv.) in anhydrous CH_2Cl_2 was added and the reaction was stirred at ambient temperature for 4 h (till the consumption of donor was seen by TLC). The reaction was then quenched by triethylamine, filtered through a Celite pad, and concentrated under reduced pressure; the resulting residue was purified by silica gel column chromatography with hexane–ethyl acetate (3 : 1) to give a pale white solid **3** (0.188 g, 0.486 mmol, 79%). The ^{1}H NMR, ^{13}C NMR, HRMS matched with data in the literature.[28]

^{1}H NMR (600 MHz, [D1] $CDCl_3$, 25 °C, TMS) δ [5.35 (dd, *J* = 10.0, 3.4 Hz), 5.31 (d, *J* = 9.8 Hz), 5.29–5.27 (m) (3H; H-2,3,4)], 5.03 (d, *J* = 1.8 Hz, 1H; H-1), 4.31–4.28 (m, 1H, H-6a), 4.28 (d, *J* = 2.3 Hz, 2H, CH_2CCH), 4.11 (dd, *J* = 12.3, 2.5 Hz, 1H, H-6b), 4.02 (ddd, *J* = 9.7, 5.2, 2.4 Hz, 1H, H-5), 2.47 (t, *J* = 2.4 Hz, 1H, CH_2CCH), 2.17 (s, 3H), 2.11 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H) (4 × $CH_3C{=}O$).

^{13}C NMR (151 MHz, [D1] $CDCl_3$, 25 °C, TMS) δ 96.23 (1C; C-1), 77.92, 75.62 (2C, CH_2CCH), 69.33, 68.97, 68.91, 66.00 (4C; C-2,3,4,5), 62.30 (1C; C-6), 54.94 (1C; CH_2CCH), 20.85, 20.73, 20.68, 20.64 (4C; 4 × $CH_3C{=}O$).

HRMS (ESI-QTOF) m/z calc. for $C_{17}H_{22}O_{10}Na^+$ 409.110 found 409.111.

2-Propynyl-1-*O*-α-D-mannose (4). To a solution of **3a** (0.188 g, 0.486 mmol) in 10.0 mL anhydrous methanol (MeOH), 0.2 mL of 0.5 N sodium methoxide (freshly prepared by dissolving sodium in anhydrous MeOH) was added. The reaction mixture was stirred at ambient temperature for 1 h, neutralized with Dowex 50WX8 (H^+) cation exchange resin and filtered. The methanol was removed under reduced pressure and trace methanol or water was removed by co-evaporation with toluene (3 × 5 mL) to give the desired product **4** as pale yellow solid (0.098 g, 0.447 mmol, 92%), which was directly used in the click reaction.

General procedure for azide–alkyne Huisgen reaction

To a solution of the pentablock copolymer (1.00 equiv.) and **4** (10 equiv.) in H_2O, aqueous solutions of $CuSO_4 \cdot 5H_2O$ (0.20 equiv.) and L-ascorbic acid (0.40 equiv.) were sequentially added. The mixture was stirred at 35 °C for 24 h. The solvent was removed under reduced pressure and residual mixture was dried under vacuum overnight.

Purification of mannose functionalized pentablock copolymers/dialysis

The residue after the click reaction was dissolved in minimum amount (<3.0 mL) of solvent (33% ethanol in water, except for PDiPAEM in which case 66% ethanol/water was used). The solution was then injected into a Slide-A-Lyzer® dialysis cassette (10 kDa M_w cutoff), and left for stirring overnight for 20 h in nanopure water. The leftover solvent inside the cassette was then evaporated under reduced pressure and the dialyzed sample was kept under vacuum overnight for complete dryness.

Phenol sulfuric acid assay

The sugar attached pentablock copolymers were dissolved in nanopure water at a concentration of 1 mg mL^{-1} and 100 μL volume per well was added to a 96 well plate. A 150 μL volume of stock sulfuric acid was added to each well following a 30 μL volume of 5% phenol. The 96 well plate was heated for 30 min at 90 °C and the absorbance was read in a microplate reader at the 490 nm wavelength.

Statistical analysis

All error bars represent 95% confidence intervals for the mean. Differences between groups were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The denoted statistical significance indicates a p-value ≤ 0.05.

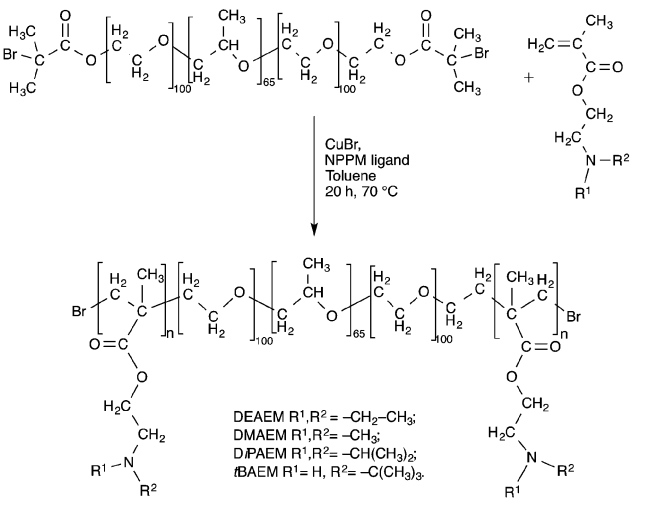
Results & discussion

The synthesis & characterization of the family of pentablock copolymers

A family of pentablock copolymers (PDEAEM, PDMAEM, PDiPAEM and P*t*BAEM) previously developed by our group as injectable drug delivery devices exhibit remarkable reversible gelation around physiological temperatures and vary in their pH-dependent micellization behavior.[2,14] These were synthesized using a Pluronic macronitiator triblock and different amine containing cationic blocks utilizing ATRP. To ensure biocompatibility, the molar mass was maintained below the 20 kDa cut off for renal excretion by the kidneys.[29] Scheme 2 is a schematic of the ATRP synthesis reaction for the four different pentablock copolymers. The pentablock copolymers were characterized by ^1H NMR and the number average molar mass (Mn) was calculated from the integral per proton ratio using the known chain length of the central triblock Pluronic. The ^1H NMR spectra and a table of molecular weights can be found in Fig. 1 and Table 1 respectively. Previous studies by our research group with the PDEAEM pentablock copolymer have demonstrated that these block copolymers form charged spherical micelles at low pHs with a hydrodynamic radius of approximately 10 nm that increase in size and transition into cylindrical micelles above a critical pH.[14,30] These polymers are potential candidates for injectable gene delivery and as such were then tested for compatibility as DNA delivery platforms.

DNA condensation & cytotoxicity studies

Agarose gel electrophoresis was used to determine the minimum concentration of pentablock copolymer required to form a polyplex based on the ratio of charged nitrogen atoms in the pentablock copolymer's tertiary amine groups and the negatively charged phosphate groups in the DNA backbone denoted as the N/P ratio. Fig. 2 shows fluorescent images of agarose gels containing the four different pentablock copolymers at various N/P ratios. In wells where the DNA did not migrate, the quantity of pentablock copolymer was large enough to neutralize the negative DNA charge, indicating the formation of polymer–DNA polyplexes. PDEAEM, PDMAEM and P*t*BAEM complexed with DNA at minimum N/P ratios of 3, 1 and 1 respectively. The PDiPAEM pentablock copolymer with the hydrophobic isopropyl groups was insoluble in water. This



Scheme 2 ATRP synthesis of block copolymer family.

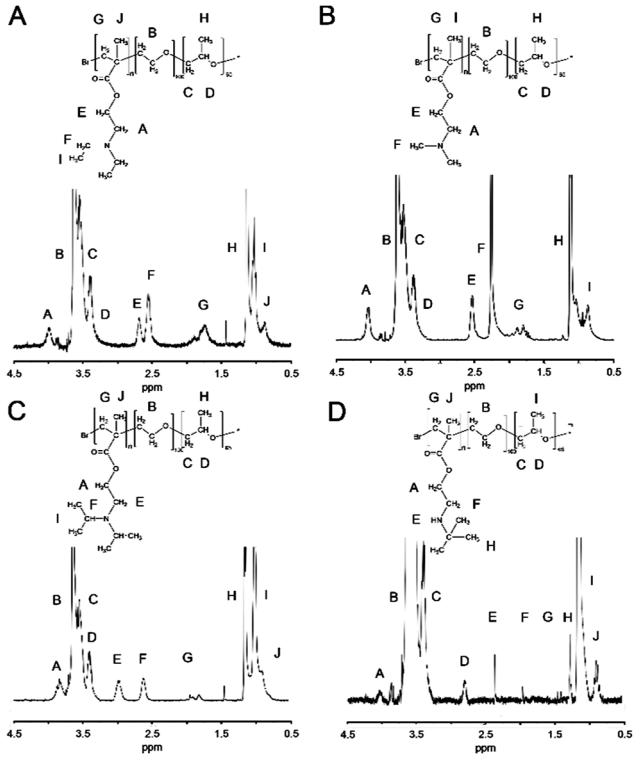


Fig. 1 ^{1}H NMR spectra of pentablock copolymers: (A) PDEAEM (B) PDMAEM (C) PDiPAEM (D) P*t*BAEM.

Table 1 Number average molar mass (M_n) and methacrylate chain length (n) calculated from ^{1}H NMR spectra of pentablock copolymers

Pentablock copolymer	M_n g mol^{-1}	n Chain length
PDEAEM	15 500	7.5
PDMAEM	14 300	4.1
PDiPAEM	13 700	2.4
P*t*BAEM	13 000	0.7

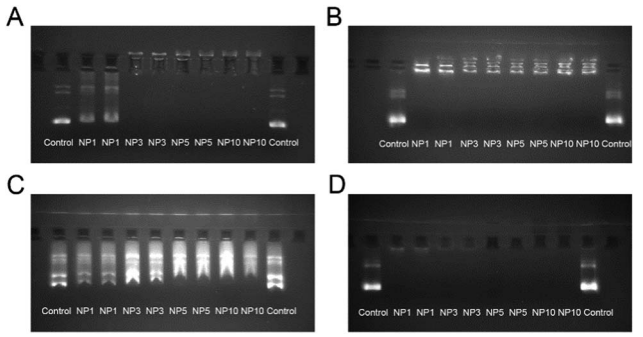


Fig. 2 Agarose gel electrophoresis of pentablock copolymer polyplexes at N/P ratios of 1, 3, 5 and 10 with a DNA only control (A) PDEAEM (B) PDMAEM (C) PDiPAEM (D) P*t*BAEM.

pentablock copolymer was unable to complex with DNA at N/P ratios less than 10, which may be attributed to the necessary sonication that was required to dissolve this block copolymer. A lower N/P ratio is desirable when designing a DNA-based vaccine formulation due to the reduction of the necessary amounts of the cytotoxic cationic groups on the polymers.

The primary obstacle that researchers developing adjuvants must overcome is toxicity, since cationic groups that can complex with DNA can be cytotoxic, which is why the cytotoxicity of the different polymers was investigated.[19,31] The cytotoxicity of the pentablock copolymers was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Four pentablock copolymers of similar molecular weights at the same N/P ratio were tested at low and high polymer concentration ranges used typically for DNA transfection studies.[17,29,32,33] Unfortunately, it was observed that the cells treated with the P*t*BAEM pentablock copolymer did not survive at high or even low concentration ranges. While the other three polymers showed low cytotoxicity, the differences between the PDEAEM, PDMAEM and PDiPAEM pentablock copolymers were statistically significant at the higher polymer concentration range (Fig. 3). The required sonication of the PDiPAEM pentablock copolymer may have contributed to the low cytotoxicity of this block copolymer.

Functionalization of pentablock copolymers with D-mannose *via* azide–alkyne Huisgen cycloaddition

The D-mannose was attached to the pentablock copolymer *via* Huisgen cycloaddition by functionalizing the polymer with an azide group and the mannose with an alkyne linker. As previously reported,[34] bromo-terminated polyacrylates can be easily substituted by the nucleophilic azide group, hence the halogen end groups of the pentablock copolymers were replaced by azide (Scheme 3). A distinct peak present between 2100 and 2300 cm^{-1} represents the azide functionalization of the pentablock copolymers (Fig. 4) which was previously absent in the non-azide functionalized polymers.

We recently developed[27] a simple, efficient thioglycoside activation protocol utilizing bismuth(V) chemistry[35,36] with a wide variety of carbohydrates containing various functional groups. This method, unlike most other current methods,[37,38] can be performed at ambient temperatures without the use of

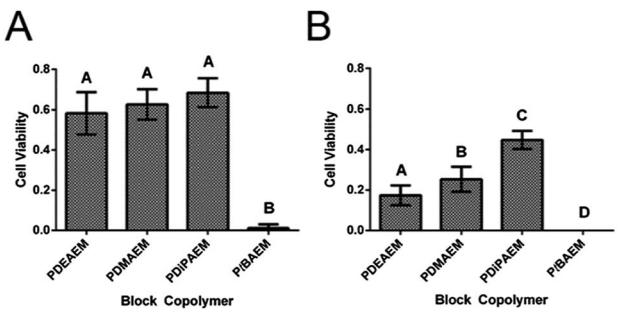
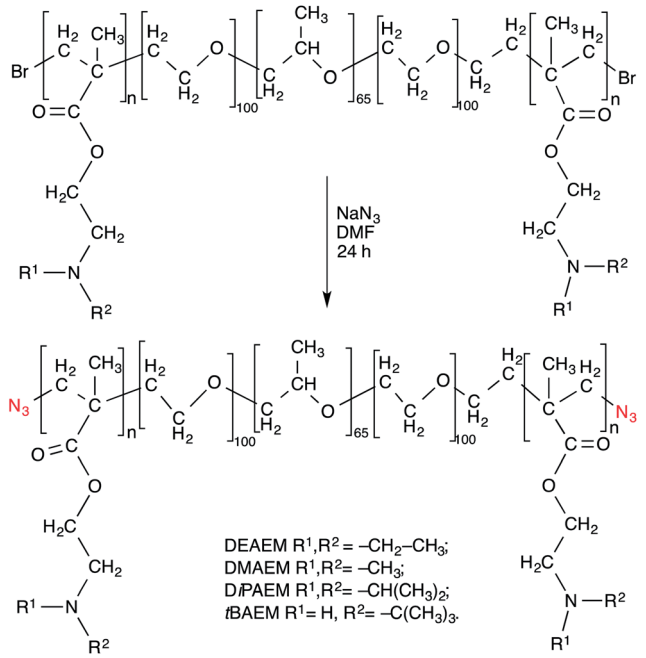


Fig. 3 The differences in cytotoxicity between the pentablock copolymers (A) a polymer concentration calculated from an N/P ratio of 20 for 0.2 μg of DNA per well. (B) A polymer concentration calculated from an N/P ratio of 20 for 0.6 μg of DNA per well. The data presented are 95% confidence intervals for the mean for four independent experiments and letters indicate statistical differences between groups where a p-value ≤0.05.



Scheme 3 Azide functionalization of block copolymer family.

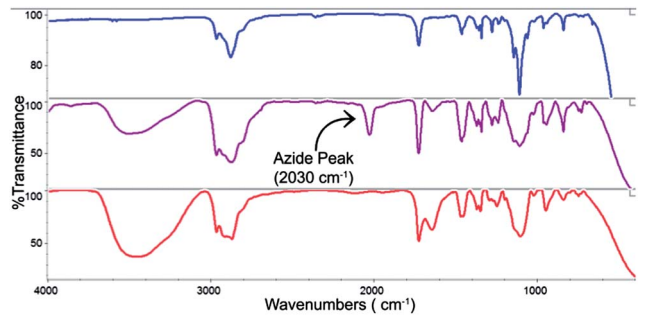
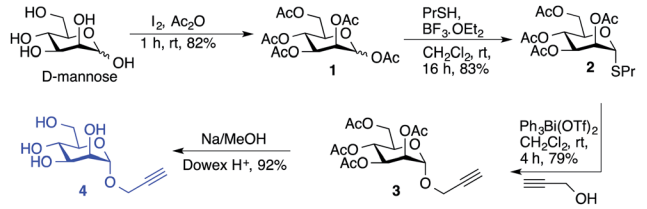


Fig. 4 IR spectrum comparison of PDEAEM before azide functionalization (top), after azide functionalization (middle) and after azide–alkyne Huisgen reaction with mannose analogue **4** (bottom).

any additives/co-promoters. Moreover, unlike many thioglycoside activation methods that rely on promoter interactions with the soft sulfur that preclude the facile use of alkenes anywhere in the glycosyl donor or acceptor, this bismuth-mediated method was shown to work effectively in the presence of alkenyl groups. In order to install the alkyne linker onto the carbohydrate, we further investigated the application of this method in the presence of terminal alkynyl systems, which like alkenes are also prone to addition by-products. The synthesis of the desired mannose analogue (Scheme 4) containing the alkyne moiety started with peracetylation of commercially available D-mannose, which was then converted to a new thiopropyl mannoside donor **2**. The donor **2** was then activated with $Ph_3Bi(OTf)_2$ in presence of propargyl alcohol to give the alkyne linked mannose **3** in 79% yield, without any interference with the alkyne moiety. Finally, **3** was deacetylated to give the final alkynyl mannose **4** in overall 50% yield in 5 steps. 1D and 2D NMR spectra of the mannose derivatives are available in the ESI.†



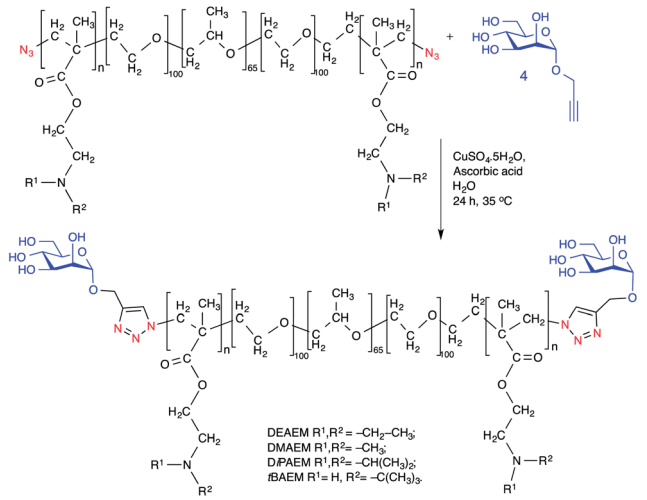
Scheme 4 Preparation of D-mannose with the alkyne linker.

After the azide and alkyne functionalization of the polymer and mannose respectively, they were coupled together *via* a Huisgen azide-alkyne click reaction catalyzed by a mixture of $CuSO_4 \cdot 5H_2O$ and L-ascorbic acid (Scheme 5).

Purification and analysis of the attachment of D-mannose to pentablock copolymers: IR spectroscopy, and phenol sulfuric acid assay

The next crucial part was to characterize the attachment as well as quantify the amount of sugar attached to the polymer. Unfortunately, the initial studies using mass spectroscopy (specifically MALDI-TOF) and ^{15}N labeled 1-D & 2-D NMR spectroscopy were not definitive. However, IR spectroscopy results showed the presence and absence of peaks, particularly a distinct azide peak (~2050–2300 cm^{-1}) (Fig. 4) before/after reaction, but did not quantify the coupling. The ^1H NMR spectra of the pentablock copolymers before and after attachment (ESI†) also shows new peaks in the region of 5–7 ppm, which are probably from the triazole ring protons and the mannose protons after the click reaction. However, since these protons are too small in number compared to the copolymer protons, they are difficult to accurately integrate to quantify the attachment.

In order to accurately quantify the amount of mannose attached to the polymer, a phenol sulfuric acid assay was carried out by dissolving the pentablock copolymers in water and



Scheme 5 Azide–alkyne Huisgen cycloaddition between D-mannose and the various pentablock copolymers.

reacting the carbohydrates with phenol in the presence of sulfuric acid (Fig. 5). Prior to these assays, the mannose functionalized pentablock copolymers were purified from the unreacted sugar/excess reagents by utilizing dialysis membrane cassettes to rule out detection of unattached sugar. The separation of the excess sugar as well as excellent coupling of the two were clearly evident in the phenol sulfuric acid assay values from before and after dialysis samples. The PDiPAEM pentablock copolymer was slightly soluble in water with vortexing but continuously precipitated out of solution at relatively low concentrations. For this reason, sonication was used for the mannose functionalized PDiPAEM pentablock copolymer for all characterization assays. Overall, it was determined that the dialyzed pentablock copolymers had approximately two moles of mannose present per mole polymer indicating that the azide–alkyne click reaction produced near-complete conversion.

DNA condensation of mannose functionalized block copolymers

We envisioned that the addition of the mannose to the pentablock copolymers should not hamper their ability to condense DNA since this ability comes from the tertiary amine groups present in the outerblocks. Nevertheless, we performed gel electrophoresis on the mannose attached copolymers to test this hypothesis. The minimum N/P ratios for DNA complexation did not change for any of the four pentablock copolymers after the azide–alkyne Huisgen reaction indicating that the attached mannose did not impact the capability of the block copolymers to condense DNA (Fig. 6).

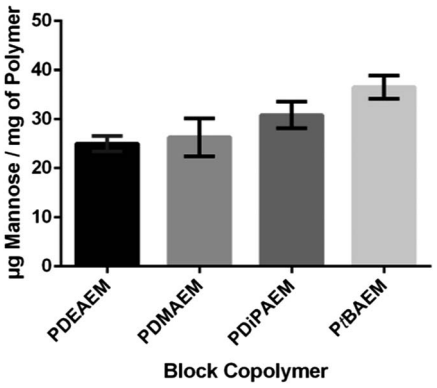


Fig. 5 Quantification of mannose functionalized pentablock copolymers. The data presented are 95% confidence intervals for the mean for four independent experiments.

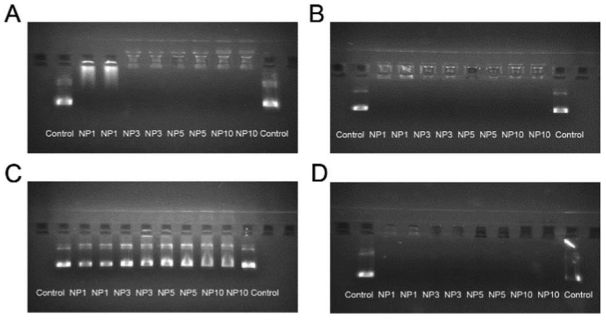


Fig. 6 Agarose gel electrophoresis of mannose functionalized pentablock copolymer polyplexes at N/P ratios of 1, 3, 5 and 10 with a DNA only control (A) PDEAEM (B) PDMAEM (C) PDiPAEM (D) P*t*BAEM.

Further discussion

Based on all the above studies, the PDEAEM and PDMAEM pentablock copolymers appear to be the optimal subunit vaccine candidates due to their low cytotoxicity and relatively small N/P ratios required for DNA condensation. The PDiPAEM pentablock copolymer was insoluble in water, requiring the use of slightly cytotoxic DMSO solvent or use of sonication, and did not complex with the DNA plasmid at relatively low N/P ratios. Although the P*t*BAEM pentablock copolymer complexed with DNA at a low N/P ratios, it proved to be highly cytotoxic.

C-type lectin receptors expressed by dendritic cells and macrophages internalize pathogens after binding carbohydrate structures on their surface.[39] Recently, a carbohydrate-functionalized polymer synthesized by Charville *et al.* demonstrated that copolymers containing a higher degree of mannose functionalization were more prone to binding lectin receptors.[40] Our method which includes the functionalization with mannose alkyne linker synthesized by bismuth(v)-mediated activation of a thioglycoside has yielded pentablock copolymers with hydrophobic polyoxyproplyene blocks that self-assemble into micelles. These pentablock copolymers are capable of condensing DNA for gene delivery through tertiary amine groups in the outer blocks.

Carbohydrate functionalized particles have been shown to enhance the expression of MHC II, costimulatory molecules and C-type lectin receptors by dendritic cells.[20] The high carbohydrate-functionalization of the pentablock copolymers may provide benefits when compared to other reported functionalized vaccine platforms, which typically focus on the decoration of particle surfaces.[41–48] The continued presence of sugars attached to polymer may provide increased and longer-lasting activation of immune cells as compared to a sugar-decorated particle, which may erode and slough off the sugars quickly.

Conclusions

Herein we report the synthesis of a family of pentablock copolymers based on Pluronic macrointiators. These four polymers were then tested as potential DNA vaccine candidates using cytotoxicity assays and their DNA complexation abilities. The block copolymers PDEAEM and PDMAEM were chosen as the lead candidates for a DNA vaccine delivery platform due to their ability to condense DNA at low N/P ratios and with relatively low cytotoxicity. All four of the block copolymers were successfully functionalized with mannose by a high-yielding azide–alkyne Huisgen reaction. In addition, the cheap, nontoxic Bi(v)-mediated thioglycoside activation method proved to be applicable to cases containing alkynes for the synthesis of alkynyl mannose in high yield. The addition of mannose to the polymer did not destroy the ability of the polymers to complex

DNA and therefore these new sugar-modified materials have the potential to activate APCs. These promising virus-mimicking nanoparticles for DNA-based vaccine delivery will be evaluated in future studies for APC activation and immune response generation.

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