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DNA-Polymer Conjugates for Immune Stimulation through Toll-like Receptor 9 Mediated Pathways

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Abstract

Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotide motifs are agonists of Toll-like receptor 9 and are currently being investigated for use as vaccine adjuvants through promotion of type I immunity. Several classes of ODN have been developed which differ in their propensity to aggregate, which in turn, alter cytokine profiles and cellular subsets activated. Although aggregation state is correlated with the change in cytokine response, it is unknown if this results from a change in the number of ODN available for binding and/or the possible engagement of multiple TLR9 molecules. Here, we examined the role of ligand valency on the activation of TLR9 through the synthesis of ODN-poly(acrylic acid) (PAA) conjugates. The compositions and size of the conjugates were characterized by UV-Vis spectroscopy, ¹H NMR, gel permeation chromatography, and dynamic light scattering. ELISA-based assays of cytokine secretion by murine-like macrophages indicate that these ODN-PAA polymer conjugates show enhanced immunostimulation at 100-fold lower concentrations than those required for ODN alone, for both TNF- α and IL-6 release, and are more potent than any other previously reported multivalent ODN constructs. Increasing valency was shown to significantly enhance cytokine expression, particularly for IL-6. Knockdown by siRNA demonstrates that these polymer conjugates are specific to TLR9. Our results define valency as a critical design parameter and polymer conjugation as an advantageous strategy for producing ODN immunomodulatory agents.

Keywords

Immunostimulation; Immunomodulation; Immune Response; DNA; Toll-like Receptor 9 Agonist

Introduction

Early detection of pathogens in humans and other jawed vertebrates relies largely on identification of non endogenous chemical structure by four protein families called the pattern recognition receptors (PRRs).[1–3] Unlike the adaptive immune systems' T cell (TCR) and B cell receptors (BCR), the germ line-encoded PRRs allow broad, quick, and

nonspecific recognition of most pathogens that is a hallmark of the innate immune system. Innate pathogen recognition is necessary for a competent adaptive immune system function, and is critical for the response to most pathogens and vaccine efficacy, but has also been implicated in the development of autoimmune disorders. [4–7] The Toll-like receptor (TLR) family, characterized by the presence of leucine-rich repeat (LRR) sequences, are the most well studied of the PRRs.[1] Toll-like receptor 9, which is expressed in humans in the intracellular compartments of B-cells, plasmacytoid dendritic cells (pDC), and neutrophils, [8] is responsible for the recognition of unmethylated CpG dinucleotides predominant in the DNA of viruses and bacteria.[9,10]

Pathogens often contain multiple pathogen associated molecular patterns (PAMPs) that are recognized by the PRRs, and PAMPs have thus been of considerable interest in understanding the mechanisms of immunity, as well as in the more practical considerations of vaccine design.[3,11,12]

Oligodeoxynucleotides (ODN) containing unmethylated CpG dinucleotides have been investigated as adjuvants for vaccines for their ability to activate the innate immune system through Toll-like receptor 9 (TLR9).[13] Four classes of ODN (A, B, C, and P) all contain unmethylated CG dinucleotides, but have different flanking nucleic acid sequences that mediate differences in their propensity to form structures through Watson-Crick base pairing,[14] have been identified. The differences in structure and aggregation of the ODNs alter not only the subset of immune cells activated by the ODNs, but also the profile of elicited cytokines.[14] These differences in activation have also been linked to expression of cellular receptors, accessory proteins, and the site of TLR9 localization, however, no definitive answer explains how DNA structure potentiates this differential response.[14–17] Elucidating the relationship between DNA presentation and the biological response to these different classes of ODN could lead to improved adjuvants as well as the ability to control immune response.

The response of TLR 9 to CpG-containing ODN is influenced by the number of CpG motifs, the backbone linkage used (phosphate or phosphothioate), and the tendency of the sequence to form secondary (intramolecular) or aggregated (intermolecular) structures. Studies directed at explaining the structural sources of this differential response have included the conjugation of CpG ODN motifs with polystyrene[18,19] or into protamine nanoparticles[20]. Other delivery vehicles for CpG ODN include positively charged lipids such as DOTAP[21], the polysaccharide schizophyllan[22], gold nanoparticles[23], carbon nanotubes[24] and a number of Watson-Crick base-paired structures[25–29]. A recent report using silicon nanoparticles demonstrates that 3' ODN conjugation promotes type I cytokines while electrostatic association promotes IFN- α secretion, providing significant insight into the role of ODN presentation on observed immunological response.[30] All of these systems permit manipulation of the spatial display of ODN, and have shown some enhancements in cytokine expression through enhanced delivery or protection from nucleases, but fail to inform how changes in DNA valency can alter activation of TLR9 by CpG ODN.

Polymeric conjugates offer an attractive alternative to such investigations. While ODN-based polymer conjugates have not previously been explored, polymeric conjugates have been used previously to study immune system processes such as B-cell signaling through the defined presentation of dinitrophenol groups[31], lectin binding by mannose-functionalized poly(HEMA)[32–34], and complement activation through BSA- and mannose-functionalized (poly methacrylate)[35]. Nucleic acids have been covalently attached to polymers for diagnostic and micro-array applications[36,37] as well as to improve the stability and delivery of siRNA[38–41]. To study the role of CpG ODN valency and size in TLR9 activation, synthetic polymer scaffolds afford interesting tools where both valency of

the ligand and size of the complex can be controlled. Here, we report the synthesis of novel, soluble ODN-poly(acrylic acid) (PAA) conjugates of controlled valency to study the activation of TLR9 in murine macrophages. Biotinylated conjugates were also produced to allow for detection by flow cytometry and confocal and transmission electron microscopy. Polymers and conjugates were analyzed by UV-VIS spectroscopy, NMR, GPC, and DLS to characterize their chemical identity and size. Flow cytometry and confocal and transmission electron (TEM) microscopy were used to analyze polymer uptake and cellular localization. The cytokine responses (TNF- α and IL-6) of the RAW264.7 murine macrophage-like cell line were examined by ELISA. We also demonstrate, through siRNA gene silencing, that the immune-stimulating properties of these novel CpG ODN-polymer conjugates are mediated by TLR9. Data indicate the high activity of these conjugates for TLR9 activation, with variations in cellular responses that correlate with macromolecular structure.

Experimental

Materials

Poly(acrylic acid) of 118K M_w and a PDI of 1.13 was purchased from Polymer Source (Dorval, Quebec) and purified precipitation in 0.1M HCl, dissolution in H₂O, filtration through a 0.22 μ m PES membrane, lyophilization, and then precipitation in ether from an acetone/isopropanol 50/50 solution. Gamma-irradiated 10K MWCO dialysis cassettes were purchased from Pierce Biotechnology (Rockford, IL) and washed with sterile filtered 30% ethanol and then hydrated with molecular grade water from Mediatech (Manassas, VA) before use. Centrifugal filtration units with a 30K MWCO were purchased from Millipore (Billerica, MA) and washed with sterile filtered 30% ethanol before use. All other reagents were used without further purification or treatment. N-(2-aminoethyl)maleimide trifluoroacetate salt (NEAM) (>95%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) (>99%), 1-hydroxybenzotriazole hydrate (HOBt) (97%), triethylamine (TEA) (99%), Dimethyl sulfoxide (99%), 4-mercaptophenol (97%), molecular grade sodium chloride, and anhydrous sodium phosphate were purchased from Sigma Aldrich (St. Louis, MO). 2-(N-morpholino)ethanesulfonic acid sodium salt (MES) (>99%) was purchased from Fisher Scientific (Pittsburg, PA). O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluoro phosphate (99%) (HBTU) was purchased from Chem-Impex (Wood Dale, IL). Biotin hydrazide was purchased from Proteochem (Denver, CO).

Oligodeoxyribonucleotide (ODN) 1668AT CG (5'-TCCATGACGTTCTGATGCTAT) and the negative control sequence ODN 1668AT GC (5'-TCCATGAGCTTCTGATGCTAT), both thiolated at the 3' end, and control sequences without the 3' C₆ thiol linker, were purchased from Integrated DNA Technologies (Coralville, IA). Sequences deviate from the published ODN 1668 sequence by the addition of an AT dinucleotide on the 3' end as a small spacer between the 1668 sequence and the C₆ thiol linker. DMEM, β -mercaptoethanol, antibiotic/antimycotic (100 $\times$), sodium pyruvate (100 $\times$), ethidium homodimer, and HEPES were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum was purchased from Denville Scientific (South Plainfield, NJ) and heat inactivated.

¹H NMR spectroscopy

All spectra were collected on a Bruker AV-400 spectrometer with a cryogenic QNP probe. NMR was performed at room temperature on samples dissolved in deuterium oxide (D₂O) or deuterated dimethylsulfoxide (DMSO-d₆), purchased from Cambridge Isotopes. For all polymer samples, 128 or 256 scans were used, with a D1 of 10 seconds and a 90° pulse program.

Synthesis of biotin-functionalized poly(acrylic acid)

PAA was dissolved at 20mg/mL in DMSO. To this solution, with constant stirring, both biotin hydrazide and HBTU, dissolved at 10mg/mL, were added to the PAA solution to yield a final molar ratio of 1:4 (biotin: HBTU). Triethylamine was added at a 10:1 molar ratio over biotin. Biotin to PAA molar ratios was 35:1 for conjugates described. After 24 hours, the reaction mixture was dialyzed twice, in a 10K MWCO dialysis cassette, against H₂O. The solution was then dialyzed twice against a 500mM NaCl solution and then a final time (twice) against H₂O. All dialysates were prepared with 18MΩ H₂O and filtered through a 0.22μm membrane and were employed at a 1:1000 volume ratio. ¹H NMR (D₂O): δ = 4.48 (bm, (C3)-CH, biotin), δ = 4.31 (bm, (C3)-CH, biotin), δ = 3.23 (bm, C-CH-S, biotin), δ = 2.86 (d, NH, biotin), δ = 2.64 (d, NH, biotin), δ = 2.29 (b, C-CH₂-C, biotin), δ = 2.09 (bp, C-CH-C, PAA), δ = 1.46–1.62 (bp, C-CH₂-C, PAA).

Synthesis of maleimide-functionalized poly(acrylic acid)

A more detailed protocol is given in the supporting information. All glassware, plastic, and reagents were treated to maintain sterility to limit product contamination by LPS. All reagents were dissolved in a 75mM MES solution buffered at pH 6.05 and filter sterilized. Poly(acrylic acid), EDC, HOBT, and NEAM were all dissolved at a concentration of 10mg/mL, and the EDC, HOBT, and NEAM were added immediately at a ratio of 1:3:4 respectively. EDC was used (6% for **118K-10** and **118K-3** and 3% for **118K-1**) of the total acid groups on the polymer respectively. The reaction was conducted under nitrogen, and purified/transferred via dialysis to the DNA reaction buffer (50 mM sodium phosphate, 11 mM citrate, 5 mM EDTA at pH 6.6 (degassed)). 4-mercaptophenol (4-MP) was reacted with maleimide groups to provide a probe for NMR analysis of the extent of maleimide functionalization of the polymers. Dialyzed polymer was lyophilized and then dissolved in D₂O for analysis via ¹H NMR spectroscopy. The extent of functionalization of the polymer with maleimide was determined by comparing the ratio of the three polymer backbone peaks δ = 2.09 (bp, C-CH-C, PAA), δ = 1.46–1.62 (bp, C-CH₂-C, PAA) to the 4 protons of the 4-MP phenyl ring δ = 6.76 (d, Ph-H(2), 4-MP), δ = 7.29 (d, Ph-H(2), 4-MP). ¹H NMR (D₂O): δ = 6.76 (d, Ph-H(2), 4-MP), δ = 7.29 (d, Ph-H(2), 4-MP), δ = 2.09 (bp, C-CH-C, PAA), δ = 1.46–1.62 (bp, C-CH₂-C, PAA).

Synthesis of ODN-functionalized poly(acrylic acid)

All apparatus and materials were treated as stated above. Oligodeoxynucleotides and DTT were dissolved in 125 mM phosphate buffer at pH 7.5 to yield an excess of DTT to ODN at a ratio of 10:1; the ODN and DTT were incubated for one hour. This ODN was then filtered, under gravity, via size exclusion chromatography using sterilized G 15 resin equilibrated in a 50mM phosphate, 13mM citrate, and 5mM EDTA ~pH 7.05 buffer, at a resin volume 10 times greater than that of the ODN solution. The eluate was fractionated and fractions tested for the presence of DNA by UV absorbance. DNA-positive fractions were combined with maleimide-functionalized polymer from centrifugal filter exchange in 50mM phosphate, 13mM citrate, 5mM EDTA ~pH 6.9 buffer. This reaction mixture of the ODN and maleimide-functional polymer components was placed in a ported bell jar, vacuum purged 3 times with nitrogen with constant stirring, and incubated under nitrogen for 5 days. The polymer control samples (PAA) were prepared from a fraction of the total synthesis, which was diluted 2-fold into 200mM pH 8 phosphate buffer and incubated for 24 hours to remove any residual maleimide via formation of the 3-hydroxyl succinimide or ring-opened derivatives of the maleimide-functionalized polymer. This control polymer, **118K**, was purified and prepared as above, without addition of ODN.

UV characterization of ODN-poly(acrylic acid) conjugates

A detailed protocol is given in the supporting information. Briefly, the 260nm absorbance values allow determination of DNA concentration by the calculated extinction coefficient ($201,800\text{cm}^{-1}\text{mol}^{-1}$ and $200,400\text{cm}^{-1}\text{mol}^{-1}$ for the CG and GC sequences respectively [42,43]); the DNA functionality of the polymer was determined by comparison of the DNA concentration (determined by UV-Vis) to the polymer concentration (determined gravimetrically). UV-Vis absorption spectra were measured on an Agilent 8452 spectrophotometer or a Thermo Nanodrop 2000.

NMR characterization of ODN-poly (acrylic acid) conjugates

Conjugate **118K-1.5** was purified in the same manner as above and then exchanged in D_2O using three, 1:80 sample dilutions. The sample was concentrated to a volume of 600 μL and characterized via ^1H NMR spectroscopy (Figure S1). The ODN 1668 CG has 22 base pairs with 32 nucleobase protons and 22 anomeric protons from the ribose. Since the ribose anomeric proton overlaps with peaks from the maleimide groups, the nucleobase protons were used for quantitation relative to the PAA backbone protons (SI).

Gel permeation chromatography

Spectra were obtained on a Waters GPC (Milford, MA) system with a 2414 refractive index detector. Phosphate-buffered saline (PBS) at pH 7.0, filtered through a 0.22 μm PES filter, was used as the mobile phase and the flow rate was maintained at 1 mL/min. Stock solutions of the polymers or conjugates were dissolved in PBS pH 7.0. Conjugates were heated to 95°C for 5 minutes to eliminate any annealing of DNA and were then flash cooled before dilution and analysis.

Dynamic light scattering

Data were obtained on a Malvern Nanoseries Zetasizer (Worcestershire, UK) and from samples in a 3mm pathlength quartz cuvette. Control samples were diluted into filtered PBS (0.22 μm PES membrane, pH 7.0) at a gravimetrically determined concentration of 0.5 μM . Samples were maintained at 20°C and equilibrated at least 5 minutes before measurement. The reported size in nm was calculated as the Z-average from the fit to the correlation function using the cumulant method with the software provided by the manufacturer. Average values from three separate samples of a given polymer are reported in Figure S2.

Cell titer blue assay

RAW264.7 cells were plated the night before the assay, in a 96-well tissue culture polystyrene plate, at 75000 cells per well. Cells were washed once with PBS and the solutions of the polymer samples were applied (at various concentrations, at a total volume of 200 μL). Samples included conjugates **118K-1**, **118K-3**, and **118K-10** as well as control wells containing the conjugate at the tested concentration but no cells. Untreated cells were plated as above and tested in the same manner without the addition of any conjugate. At 8 hours the cell titer blue reagent (Promega Madison, WI) was added to all wells for testing and incubated for 2 hours at 37° C. The supernatant was removed from the wells and placed in a 96-well plate. Samples were characterized at a fluorescence emission wavelength of 600nm using a Perkin Elmer VICTOR₃V multilabel counter (Perkin Elmer; Waltham, MA).

Cell culture

RAW264.7 cells were cultured on tissue culture polystyrene plates (Cell Treat; Shirley, MA) in DMEM supplemented with 10 mM HEPES, 55 μM β -mercaptoethanol, antibiotic/antimycotic, and sodium pyruvate (Invitrogen), and 10% fetal bovine serum in an

environment containing 5% CO₂. Cells were used up to passage 8 and maintained at between 20 and 90% confluence. Cells were washed with calcium- and magnesium-free PBS and scraped for passage.

Bone Marrow Derived Macrophages (BMDM)

BMDM were prepared as previously described[44] and isolated from femurs of BALB/C mice and cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM GlutaMax-1, 100 U/ml penicillin, and 100 µg/ml streptomycin in the presence of murine GM-CSF (5 ng/ml). At day 4, nonadherent cells were collected and cultured for a further 3 d in GM-CSF (5 ng/ml). On day 7, adherent cells were harvested and plated in cell culture 96-well plates at 200,000 cells/mL (50,000 cells/well).

Flow cytometry

A detailed protocol is given in the supporting information. Briefly, three time conditions were analyzed to determine if the polymer conjugate was being actively endocytosed. RAW264.7 cells were exposed to conjugates **118K Biotin-1** and **118K Biotin-10** for 20, 60, and 120 minutes, or at 4°C for 120 minutes, and prepared as described in the SI. A separate experiment shown in the SI compares active and passive endocytosis (37°C permeabilized vs. 4°C permeabilized respectively), with the extent of surface labeling (37°C permeabilized vs. 37°C non-permeabilized for intracellular versus extracellular labeling, respectively). Ethidium homodimer was used to identify dead cells excluded from analysis. Cell detection was conducted on a BD LSR II cell analyzer (BDBiosciences; San Jose, CA) with gates set by untreated cells; a total of 10,000 events were recorded. Flow analysis was conducted using FlowJo software (Tree Star; Ashland, OR).

Confocal microscopy

A detailed protocol is given in the supporting information. Briefly, RAW264.7 cells were seeded at 150,000 cells per chamber in Nunc 8-chamber microscopy slides (Nunc; Rochester, NY). Cells were washed once with PBS prior to use and then treated with 200 µL of a 2 µM solution of FM 4–64 FX (Invitrogen; Grand Island, NY) in HBSS for 4 min at 37°C. Cells were then aspirated and treated with 200 µL of either polymer **118K Biotin** or conjugate **118K Biotin-10** for 12 min at 37°C for active endocytosis in the presence of the plasma membrane localizing FM4-64. Cells were fixed, permeabilized, blocked, treated with a 10nM solution of the Qdot 525 streptavidin conjugate for one hour to label the biotinylated scaffold, and nuclei stained with a 2.5µM concentration of DRAQ5 (Biostatus Limited; Leicestershire, UK). Cells were aspirated and placed in PBS for imaging. Imaging was conducted using a 780 Zeiss confocal microscope (Carl Zeiss Microscopy; Thornwood, NY) with a 40 × water lens (1.2 NA) with all images 1056×1056 pixels. The image in Figure 4 a 2-dimensional representation of a 30 image Z-stack with the image LUTs adjusted to min/max and interpolated by Zen (Zeiss Oberkochen, Germany). Image analysis was conducted by colocalization mask analysis using pixel overlap intensity thresholds to limit colocalization counts to only spots manually identified as positive to generate an image mask (Figure S3, Zen). The total number of colocalization spots for each mask was then counted using the Velocity software package (Perkin Elmer; Waltham, MA).

Transmission electron microscopy

A detailed protocol is given in the supporting information. Briefly, RAW264.7 cells were lifted, counted, and divided into 15 mL conical tubes at 375,000 cells/mL per tube in 500 µL of DMEM containing 50nM biotinylated polymer conjugate **118K Biotin-10** or non-biotinylated polymer conjugate **118K-10** prehybridized for 10 minutes with 0.5nM streptavidin conjugated 10nm gold nanoparticles (Electron Microscopy Sciences; Hatfield,

PA). Cells and streptavidin gold-biotinylated polymer conjugate and control were incubated for 1 hour at 37° C and 5% CO₂ with occasional shaking to keep cells suspended. Cells were then washed and fixed in 100mM sodium cacodylate, 2% glutaraldehyde, and 2% paraformaldehyde pH 7.4 (Electron Microscopy Sciences; Hatfield, PA) for 1 hour at room temperature for TEM sample prep. Cells are additionally fixed in osmium tetroxide, embedded in resin with Embed-812 (Electron Microscopy Sciences, Hatfield, PA), and stained with uranyl acetate. TEM images were collected with A Zeiss LIBRA 120 transmission electron microscope (TEM) is an energy filtering TEM equipped with a Gatan Ultrascan 1000 2k×2k CCD camera.

Response of RAW264.7 macrophages to stimulatory ODN-poly(acrylic acid) conjugates

RAW264.7 cells were seeded the night before the experiment, at 75,000 cells/well in a 96-well tissue culture plate. Media was removed from plates and the wells washed with sterile PBS before addition of the solutions containing the conjugates. Conjugates were heated, directly before addition to the cells, to 95°C for 5 minutes, flash cooled, and diluted into media as described above at various concentrations and a final volume of 200 µL per well. Conjugates were added to the cultured cells, and after 8 hours, the supernatant was removed and frozen.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed by analysis of supernatants collected from experiments described above, diluted with 10% FBS in PBS. Monoclonal antibody sets for murine IL-6 and TNF-α were purchased from Becton Dickinson (BDbiosciences; San Jose, CA), and used with adherence to the provided protocol. Independent values are from two independent measurements at two different concentrations within the linear range of the standard curve. ELISA measurements were measured using a Perkin Elmer VICTOR₃V multilabel counter (Perkin Elmer; Waltham, MA).

siRNA transfection

Transfection was accomplished using the Qiagen HiPerFect reagent and protocol. Briefly, RAW264.7 cells (75 µL) were plated in a Costar 48-well cell culture plate with a cell concentration of 2,000,000 cells/ mL (150,000 cells per well) in culturing media before transfection. TLR9-specific siRNA sc-40271 or control siRNA sc-37007 (Santa Cruz Biotechnology Santa Cruz, CA) was diluted to a concentration of 100 nM in 75 µL OptiMem (Gibco), with addition of 3 µL HiPerFect and mixing by vortexing. Samples were incubated for 5–10 minutes at room temperature. After incubation, the siRNA mixture was added onto cells and mixed by pipetting 3 times. Samples were incubated for 5 hours under cell culturing conditions and then were supplemented with 300 µL culturing media. Stimulation, as described above, was started 24 hours later.

RT-PCR

The siRNA KD of TLR9 was checked by RT-PCR. Detailed protocols are given in the supporting information.

Statistical analysis

Statistical analyses were calculated using Kaleidagraph (Synergy Software; Reading, PA); statistical significance was calculated with one-way ANOVA with a post hoc Tukey's analysis at the 99% confidence level. A minimum of three independent samples were used for analysis.

Results and Discussion

Design and synthesis of poly(acrylic acid)-DNA conjugates

It is known that differential cytokine responses are demonstrated by ODNs with differing aggregation, which offers opportunities to tailor the responses of cells of the immune system. The use of polymer scaffolds, in particular, allows the design of novel materials with greater control over presentation and number of ODN ligands than that previously possible with ODN aggregates or complexes. Because TLR association with lipid rafts[32,34] and receptor colocalization are important for the formation of cytosolic signaling protein scaffolds,[45] we postulated that control of the number and density of ODN ligands available for interaction with TLR9, in particular, would be a good target for affecting cytokine response. The design of polymeric carriers for such differential display of ODN ligands was based on several considerations. Although positively charged polymers such as poly(ethylene amine) have been widely used as nucleic acid carriers, in order to promote condensation and protection of the nucleic acid, the passage of the nucleic acids through cellular membranes into the cytosolic space is mediated at least in part through the interactions of the positively charged polyplex with the negatively charged cellular membrane[46–48]. Such nonspecific interaction of the conjugates with cellular membranes is undesirable in the current studies, as the goal is to probe the role of ODN display in the TLR9-mediated activation of immune cells without complications of interactions with the polymer scaffold. Poly(acrylic acid) (PAA) was thus chosen as the scaffold owing to its high negative charge at physiological pH, as well as the fact that low-PDI polymers are available over a large range of molecular weights. The high negative charge density of both the polymer and the nucleic acid also serves to prevent macromolecular interaction or aggregation of the ligand and polymer. PAA polymers of a molecular mass of 124kDa were employed in these studies; these polymers exhibit a hydrodynamic diameter of approximately 35nm under physiological conditions, as assessed via dynamic light scattering (Figure S2), providing an intermediate size scaffold (between monomeric ODN and other aggregate-based particles) for investigation, and dimensions that are sufficiently large that engagement of multiple TLR receptors is possible.[49–51]

PRRs can detect very low concentrations of PAMPs[52] so a sensitive imaging strategy was necessary in the present work to ensure detection of synthesized PAA conjugates by flow cytometry and confocal microscopy. Multiple biotin labels were thus employed to enhance the binding capacity of the PAA conjugate for streptavidin-labeled fluorophores. Biotin was chosen for its versatility for labeling of the polymer with any streptavidin-functionalized detection agent, as well as for its small size, which minimizes any steric impact on the binding of the conjugate to TLR9. Similar strategies have been employed in other work to provide high avidity binding of DNA to solid substrates for DNA hybridization,[53] as well as enhancing uptake of PAMAM-biotin conjugates in an ovarian cell cancer line[54]. Biotinylation was accomplished through the chemical protocols detailed in the experimental section and shown in Scheme 1, the extent of biotinylation determined via ^1H NMR, and functional streptavidin binding by HABA displacement assay (Supporting Information). The effect of biotinylation on the activation of RAW264.7 cells (via assessment of TNF- α expression) was monitored to ensure that the modification of the polymer did not complicate interpretation of cellular uptake and activation. Based on these activation results (Figure S4), a biotin functionality of approximately 30 showed minimal activation with full binding accessibility, and was thus chosen for labeling and detection in flow cytometry and confocal microscopy experiments.

Synthesis of the ODN-PAA conjugates required a robust and efficient bioconjugation strategy compatible with available chemical end-functionalities available for synthetic ODN. We chose to functionalize PAA with N-(2-aminoethyl)maleimide (NEAM) through the

chemical protocols outlined in Scheme 1 and subsequent reaction with a 3' thiol-functionalized ODN. Similar chemical approaches were employed by Lifland and colleagues for the synthesis of poly(ethyleneglycol)-ODN hybridization probes.[39] ODN must be conjugated through the 3' end for proper interaction with TLR9[55] and thus ODN with a 3' C6-thiol linker were employed in these studies. Synthesis of the PAA-ODN conjugates described in Table 1 was accomplished by varying the molar ratio of 3'-thiol ODN to maleimide-functionalized PAA synthesized as described in Scheme 1 (lower pathway **b**). The buffers used for both the column purification of the reduced 3'-thiol ODN and for the maleimide-functionalized PAA were optimized to reduce hydrolysis of the maleimide group and maintain the nucleophilicity of the thiol group. Small changes in pH or ionic strength resulted in drastic changes in observed reaction efficiency, often with no observed product. Both TCEP and DTT were examined as reductants for the 3' thiol-functionalized ODN. Only DTT, with subsequent purification via SEC with a Sephadex G 15 column and 50mM phosphate, 13mM citrate, 5mM EDTA ~pH 7.05 buffer, yielded efficient conjugation. Table 1 indicates the maleimide functionalities of the conjugates used in these studies, which ranged from 10 to 45 and yielded 1 to 10 ODNs per polymer. Polymer conjugates **118K Biotin-1** and **118K Biotin-10** were synthesized with both biotin and maleimide functionality, for visualization of these polymer conjugates during flow cytometry and microscopy, while **118K-1**, **118K-1.5**, **118K-3**, **118K-10GC**, and **118K-10** were synthesized (without biotin) for cellular activation studies, as shown in Scheme 1. Polymer conjugates **118K Biotin-1**, **118K-1.5**, and **118K-1** were synthesized with a lower maleimide functionality of approximately 10 (Table 1) owing to the fact that higher maleimide functionalities introduced slight absorbance at 260nm that interfered with the spectrophotometric determination of ODN functionality for the PAA-ODN conjugates equipped with a single ODN (data not shown). The higher functionality polymer conjugates **118K Biotin-10**, **118K-10**, and **118K-3** were synthesized from a PAA equipped with a higher maleimide functionality of approximately 45 (Table 1) for more efficient conjugation and higher ODN functionality.

Purification methods were also optimized to allow complete removal of unreacted ODN from any HMW species (>118K). Purification buffer with an elevated pH (8) was employed to ionize all phosphates and carboxylic acids, thus maximizing electrostatic repulsion of the ODN from the polymer scaffold. In addition, the expected covalent bond formation was validated (in part) by examining several buffer conditions (over several different syntheses) that would disrupt noncovalent association of ODN with the PAA polymers and conjugates. Various solutions (1M NaCl (ionic forces), 18 MΩ H₂O (hydrophobic forces), and 5% DMSO (also hydrophobic forces)) were tested as eluents; there was no decrease, after five washes under these various conditions, in the observed ODN absorbance at 260 nm of the high molecular-weight fractions. Analysis of all reactions and fractions during the synthesis confirmed that all ODN was accounted for during the synthesis, and that there was no alteration of the expected UV absorbance properties. Thus, the ODN absorbance at 260nm was employed to indicate the concentration of ODN in a given sample, and the functionality of the polymer determined by using the absorbance value and the pre-measured mass of polymer in a given sample.

To validate that these protocols accurately represented the ODN functionality of product given conjugate, ¹H NMR was employed. The polymer 118K-1.5 was used to compare ODN functionalities determined via UV-Vis and ¹H NMR. The low ODN:polymer ratio of this sample provided the most stringent test case as samples with low numbers of ODN pendant ligands are the most susceptible to interference in the UV spectra. The functionality value determined by both methods was shown to be in very close agreement, with UV-Vis analysis yielding a valency of 1.5 and ¹H NMR analysis yielding a valency of 1.6. Also, the NMR spectra (Figure S1) shows peak broadening not only for the polymer proton peaks, but

also for the ODN peaks (when compared to unreacted ODN (not shown)) as a result of slower tumbling, and an indication of the conjugation of the ODN to the polymer. The inset of Figure S1 shows the UV trace of this conjugate demonstrating the characteristic peak in absorbance at 260nm.

In addition, all raw materials and final polymers and conjugates were tested, via LAL assays, for lipopolysaccharide (LPS), a ubiquitous environmental contaminant from Gram (–) bacteria, to confirm that there would be no activation of the RAW264.7 cell line owing to contamination of the ODN or polymer samples. All synthesis protocols were designed specifically to prevent LPS contamination and all conjugates were measured to be <0.02 EU/mL; these values are well below levels capable of activation (data not shown).

Characterization of ODN-PAA conjugates—Gel permeation chromatography (GPC) analysis was used to determine if the ODN and polymer co-eluted, which would further indicate covalent association and indicate the absence of monomeric ODN in any polymer conjugates. We anticipated that the small size of the ODN (~2 nm)[56] would not significantly modify the hydrodynamic volume of the much larger polymer (~35 nm). Figure 1 shows that the separate peaks (detection at 260nm) resulting from the free ODN (open circles) and the polymer-ODN conjugates **118K Biotin-10** (light grey crosses), **118K-1** (black diamonds), and **118K-10** (closed circles) are clearly discernible, supporting that any absorbance of the high molecular mass peak at the polymer elution time arises from ODN that is covalently conjugated to the polymer backbone. A slight contamination of free ODN in sample **118K-10** (ca. 2%) is observed; this low concentration of monomeric ODN was separately determined to be nonactivating to the macrophage-like cell line, and the **118K-10** sample was thus employed in cellular assays without further purification. Solutions of conjugates **118K Biotin-10**, **118K-1**, and **118K-10** were prepared gravimetrically to yield solutions with similar ODN concentration; the similar observed intensity of the absorbance from these samples thus further confirms the accuracy of the UV-Vis analysis. The results of this GPC analysis, which illustrate that all polymer conjugates, regardless of valency, exhibit the same hydrodynamic volume as the unmodified PAA, when taken together with the results of the DLS (Figure S2), suggest that all of the PAA-ODN conjugates are approximately 35 nm in diameter.

The cytotoxicity of conjugates **118K-1**, **118K-3**, and **118K-10** was monitored using the cell titer blue reagent, which measures the reductive capacity of metabolically active cells, and thus indirectly measures cell viability. The data in Figure 2 present results for RAW264.7 cells exposed to the highest concentration of conjugate tested (normalized to 200nM DNA), and illustrate that the metabolic activity of each sample is at or above the level of the control sample. Thus, no cytotoxicity was observed for any of the conjugates tested; a slight increase in metabolic activity measured for the cells exposed to **118K-10** is likely a result of innate cellular activation of TLR9 by the conjugate.[28]

Flow cytometry and microscopy analysis of uptake and localization—Flow cytometry, as well as confocal and transmission electron microscopy, were used to study the uptake and cellular distribution of conjugates, respectively. The flow cytometry studies were designed to determine whether the cellular uptake was an active process and the microscopy studies to assess if the localization within the cell was defined and in membrane-bound vesicles. To this end, we employed **118K Biotin-10** and **118K Biotin-1** conjugates, which allowed detection via the fluorescence of streptavidin-labeled quantum dots. The flow cytometry data are shown in Figure 3, and the data illustrate the levels of cellular internalization of **118K Biotin-1** and **118K Biotin-10** at 37°C over a time course of 20, 60, and 120 min, and at 4°C after 120 min, in order to assess if uptake is an active or passive process. There is clearly an increase in the mean fluorescence intensity (MFI) over time for

both **118K Biotin-1** and **118K Biotin-10** ($p < 0.001$) at 37°C with only a small detectable increase in MFI at 4°C over 120 min ($p < 0.0001$). With the increase in MFI observed by flow cytometry only for samples incubated at physiological temperature, we conclude that internalization of these conjugates is an active process. We also employed a separate flow cytometry experiment (Figure S5) in which permeabilization conditions were altered to permit labeling only of the surface of the cell. Specifically, the fluorescence of permeabilized and non-permeabilized cell populations (at 37°C) were compared, and the fluorescence of the non-permeabilized cells at 37°C was compared to that of permeabilized cells at 4°C . If uptake is an active process, the permeabilized cells incubated at 37°C should show an elevated fluorescence relative to the other two conditions. The data in Figure S5 clearly illustrate this, and also illustrate that the histograms for **118K-1** and **118K-10** for the permeabilized 4°C and non-permeabilized 37°C conditions overlay, thus indicating that any fluorescence observed at 4°C is a result of surface labeling. Taken together, these two flow cytometry experiments clearly confirm the active internalization of the PAA-ODN conjugates.

Confocal microscopy (Figure 4a) was used to corroborate the flow cytometry data (that the conjugates were internalized and not simply associated with the plasma membrane), but more importantly, to show that conjugates had defined distribution throughout the cell and were localized within membranous structures and not dispersed in the cytosol. The dye FM4-64 was used by Lande et al. to determine endosomal colocalization of DNA complexed by the cathelicidin LL_37 [57]. Similarly, the fixable analogue FM4-64FX was used in the present study to mark membrane-mediated endocytic events and internalization of the polymer conjugate **118K Biotin-10** (or polymer **118K Biotin**). Intracellular colocalization of the dye with quantum dots would indicate that the dye and conjugate were concurrently endocytosed from the plasma membrane. Figure 4a shows a representative confocal image with the green indicating the Qdot 595-complexed **118K Biotin-10**, the red indicating the FM4-64, and the blue indicating DRAQ5-stained nuclei. The distribution of **118K Biotin-10** is limited to localized regions of 150–500nm, which corresponds to the expected size of endocytotic vesicles.[58,59]. This colocalization was further analyzed via intensity-based analysis of image masks (example Figure S3) on Velocity software, to estimate the number of colocalization events (Table S1). The scaffold **118K Biotin** exhibited $2\frac{1}{2}$ times the number of colocalization events of **118K Biotin-10**, perhaps from greater accessibility of the pendant biotin or a difference in receptor-mediated endocytosis. Although these data show that the biotin-streptavidin detection method is likely only qualitative, they unequivocally demonstrate colocalization of the Qdot-labeled conjugates with the plasma membrane-localized FM4-64 and thus demonstrates that the scaffold is endocytosed from the plasma membrane.

Transmission electron microscopy (TEM) was used to corroborate that the polymer conjugates were indeed localized within endocytotic vesicles. Biotinylated **118K Biotin-10** (or the non-biotinylated **118K-10**) was mixed with streptavidin-gold nanoparticles (at a 100:1 polymer:particle mixing ratio in order to limit labeling of the polymer scaffolds to a single (or a very low number of) gold nanoparticle(s)) prior to addition to cells. Figure 4b shows a representative image of a cell from the **118K Biotin-10** samples and clearly shows the gold-labeled **118K Biotin-10** within two distinct membrane-bound endocytic compartments with sizes appropriate for early or late endosomes and matching those in Figure 4a. Twelve independently selected cells were imaged for both sample and control, and the number of endocytosed gold particles counted. Analysis demonstrates the number of endocytosed gold particles in the cells treated with the biotinylated **118K Biotin-10** (28 ± 20) is statistically different ($p = 0.00071$) from the number of nanoparticles in cells treated with the non-biotinylated **118K-10** control (5 ± 5), indicating that the uptake of the gold nanoparticles is mediated by uptake of the biotinylated, ODN-modified polymer, rather than

by aggregates of free gold nanoparticles. The conclusion that gold nanoparticle uptake is mediated by the polymer scaffold is further substantiated by the fact that in the **118K Biotin-10** samples, which can bind to the gold particles through the biotin-streptavidin interaction, no gold nanoparticle aggregates were observed. In contrast, aggregates were common in **118K-10** samples in the absence of biotin-streptavidin interactions, owing to the high aggregation propensity of free gold nanoparticles (data not shown). The combination of the flow cytometry, confocal microscopy, and TEM data clearly illustrate that the ODN-modified scaffold is actively internalized and that endocytotic events localize conjugate within vesicular compartments. The microscopy images also show that the conjugates are localized in defined, vesicular-sized areas within the cell; the lack of diffuse cytoplasmic distribution indicates the lack of endosmolytic breakage.

RAW264.7 cell-line response to ODN-PAA conjugates—Murine-derived

RAW264.7 cells are the standard cell-line for TLR9 studies due to their expression of TLR9 and their macrophage-like characteristics.[23,26,60] These cells were cultured and exposed to ODN-PAA conjugates of multiple valencies and the cell supernatants were analyzed by ELISA for murine TNF- α and IL-6. These two cytokines are both type I inflammatory cytokines and are secreted by cells in response to TLR9 activation by CpG motifs in pathogen DNA.[10] Figure 5 shows the TNF- α and IL-6 responses, at 8 hours, to free ODN (Figures 5a and 5b respectively) compared on the same scale to the cytokine responses to polymer conjugates **118K-1**, **118K-3**, **118K-10** (all normalized to ODN concentration), and controls (PAA, monomeric ODN, monomeric GC-ODN, and a GC-ODN-containing PAA) (Figures 5c and 5d). The controls include the polymer conjugate **118K-10GC** (comparable in construction to **118K-10**), equipped with the control ODN sequence 1668GC that is not competent for TLR9 activation; this conjugate shows no cytokine expression above the PAA controls. Both Figure 5a and 5b show the expected increased cytokine excretion with increasing free ODN concentrations (1 μ M, 2 μ M, 6 μ M, 7 μ M, 8 μ M, and 10 μ M). Figures 5c and 5d show the cell response to conjugates **118K-10**, **118K-3**, and **118K-1** (at ODN concentrations of 16.6nM, 100nM, and 200nM), and illustrate the lack of cytokine secretion from cells exposed to the negative ODN-PAA control (GC motif), the unmodified PAA, or the monomeric ODNs at these concentrations.

The TNF- α response (Figure 5c) shows an increase in cytokine secretion with increasing conjugate concentration for all samples in this concentration range, indicating the sensitivity of the cells to the polymer-bound ODN. The data also show that the conjugate **118K-1** (with a single polymerbound ODN) elicits greater TNF- α production relative to that elicited by the free 1668 CG ODN. This could result from higher resistance to exonucleases activity for the polymer-bound ODN conjugate, consistent with the fact that exonucleases degrade DNA from the 3' terminus,[14] and that this terminus was used for conjugation of the ODN to the PAA scaffold. Increased cellular uptake could also be the source of this enhancement, however, the labeling strategy used here does not allow direct comparison to single ODN in flow cytometry and confocal analysis. A modified detection strategy is currently being used to address the origin of increased TNF- α production in response to the **118K-1** conjugate. Both the **118K-10** and **118K-3** polymer conjugates show statistically significant increases in TNF- α production over **118K-1** at ODN concentrations of 100nM ($p = 0.003$). The **118K-10** polymer conjugate also shows a statistically significant increase in cytokine release (TNF- α) compared to that of **118K-1** at an ODN concentration of 200nM (11605 ± 3650 pg/mL vs. 5599 ± 4960 pg/mL; $p = 0.02$), indicating that higher valencies elicit increased cytokine responses normalized for ODN concentration. Although the detection method employed here is not quantitative, it is likely that the higher-valency scaffolds have lower intracellular concentrations than the low-valency scaffolds, given the necessary differences in the scaffold concentrations required to normalize ODN concentration. Thus, the higher valency

scaffolds may be more potent activators of TNF- α production than indicated by these data. Current studies are focused on determining relative intracellular concentrations for conjugates of differing valency. Regardless, comparison of the DNA concentrations required to elicit similar cytokine responses (for the conjugates versus the free ODN) suggests that the conjugates are at least 100-fold more potent for cytokine production and that this potency is related to valency.

Comparisons of Figures 5b and 5d illustrate that the enhancement in IL-6 expression for the conjugates relative to the free ODN is perhaps even more striking. Here, enhancement in cytokine production by the polymer conjugates is highly dependent on the ODN valency (all samples are normalized to ODN concentration) and both **118K-3** and **118K-10** show significant increases in immunostimulation as indicated by IL-6 production. Conjugate **118K-10** (482 ± 165 pg/mL) shows statistical significance over **118K-3** (179 ± 38 pg/mL) and **118K-1** (13 ± 6 pg/mL) ($p < 0.0001$) and **118K-3** shows significance over **118K-1** ($p = 0.0148$). Comparisons of the IL-6 production between the free ODN and conjugates (Figures 5b and 5d) show that conjugates **118K-3** and **118K-10** elicit the same (or even 2-fold greater) IL-6 response as a free ODN at 100-fold lower concentrations (100 nM vs. 10 μ M). We are currently exploring the biological implications of this altered cytokine profile.

To ensure that the enhanced immunostimulation of these novel ODN-polymer conjugates is indeed mediated through TLR9 and not other PRRs, we employed siRNA to silence TLR9 mRNA and evaluate the effect of knockdown on RAW264.7 stimulation (Figure 6). The TNF- α concentrations in the media were analyzed via ELISA for untreated (dark grey), TLR siRNA-treated (white), and mock siRNA-treated (light grey) cells exposed to ODN 1668 (2.5 μ M), conjugate **118K-10** (200 nM in ODN), and LPS (100 ng/mL) (Figure 6). As is illustrated in the data, treatment of the cells with TLR9-silencing siRNA results in an over 90% decrease of the observed TNF- α response versus that observed for untreated cells, for both ODN 1668 and **118K-10** ($p < 0.01$); no such decrease in TNF- α response was observed for the mock samples. We also monitored knockdown by RT-PCR, confirming that siRNA treatment also significantly lowered TLR9 mRNA levels in stimulated cells (Figure S6). These data pinpoint TLR9 as the immunological target of these novel ODN-PAA conjugates.

As a further test of the utility of these PAA-ODN conjugates, we have confirmed the immunostimulatory impact of these novel ODN-PAA conjugates on GM-CSF-matured, murine bone marrow-derived macrophages (BMDM). A titration of the ODN 1668 (panel a) was used to compare the levels of IL-6 expression in BMDM treated with monomeric ODN to that of cells treated with the conjugate **118K-10** (Figure 7). Panel (a) illustrates the levels of IL-6 production for GM-CSF matured macrophages exposed to various ODN concentrations (1 to 10 μ M), as assayed by ELISA. Panel (b) shows the concentrations of IL-6 produced by GM-CSF matured macrophages exposed to conjugate **118K-10** (dark grey), ODN 1668 (light grey), and the control ODN 1668 GC (white) at ODN concentrations of 200 nM, 100 nM, and 6.3 nM. As illustrated via comparisons of the IL-6 production, conjugate **118K-10** elicits similar IL-6 expression at a concentration approximately 10-fold lower than the monovalent ODN 1668 (Panel b). These results validate, in primary macrophages, the enhanced immunostimulation of the multivalent ODN-PAA conjugates suggested by results in the RAW264.7 cell line.

When comparing the activity of these ODN-PAA conjugates to that of other complex displays of DNA described in the literature, conjugates **118K-3** and **118K-10** elicit a greater fold increase over monomeric ODN of TNF- α , than any other complex of comparable concentration. Rattanakit et al. demonstrate a 15- to 20-fold increase in TNF- α (at 8 hours) for a 12-functional hybridized DNA dendritic structure,[26] while **118K-10** shows an 35-

fold increase at comparable concentrations. Nishikawa et al. and Li et al. observed 6- and 15-fold increases for 3-functional Y or tetrahedral-structured displays,[28,60] while **118K-3** demonstrates a 85-fold increase at comparable concentrations. It is also important to note that the relative increases of TNF- α production in our studies are greater than those of other reports, despite the fact that the ODN employed in our studies is significantly less potent than those used in the above reports (which contain multiple CG motifs).[61] Taken together, comparison of our data with these previous studies illustrates the substantially enhanced potency of these simple ODN-PAA conjugates versus any other multivalent ODN displays yet reported.

The IL-6 results from our studies (8 hours) are difficult to compare directly with other published results, due to the fact that IL-6 is commonly assayed at later time points (24 or 36 hours) because its ODN-induced expression is kinetically slower compared to that induced by other PRR stimuli.[62] Despite the earlier time point utilized in our studies (8 hours), we observe 100-fold increases in IL-6 expression (multivalent vs. monomeric) for the conjugate **118K-10** compared to the previously reported 5-fold increase observed for a 12-functional dendritic structure.[26] Conjugate **118K-3** shows a 30-fold increase, compared to a 10-fold observed by Nishikawa et al. and an approximately 20-fold increase observed by Li et al. for ODNs at comparable concentrations.[28,60] We have preliminary data (not shown) suggesting that the production of IL-6 at 24 hours is increased over 1000-fold for both **118K-3** and **118K-10** at 100nM, values much greater than those of any previously reported immunostimulatory complexes. The most critical point of comparison is the change in IL-6 expression observed with increasing valency for the ODN-PAA conjugates. The IL-6 concentrations elicited by **118K-3** are at least 13-fold greater than those elicited by **118K-1** at all ODN concentrations, while enhancements reported previously by Li et al. for a 3-functional structure are only 1.5 fold greater than the monofunctional structure.[28] We observe IL-6 concentrations 2.5-fold greater for cells stimulated with **118K-10** versus **118K-3** (even at normalized ODN concentrations), further highlighting the importance of ODN valency. Taken together with previously reported studies, the data reported here illustrate that, at the very least, expression of IL-6 occurs more rapidly in cells exposed to PAA-ODN conjugates of multiple valency than is commonly reported for other multivalent DNA displays.

These PAA-ODN conjugates represent useful probes of biological function that may inform the design of potential therapeutics. As introduced above, no non-aggregated, polymer-based ligands have been previously used to study TLR9 ligand recognition. A significant advantage of the polymer-based approaches versus those based on aggregation or DNA-origami (in addition to the demonstrated increased immunostimulation that the polymer carriers provide), is the substantially enhanced versatility to control both carrier size and valency; this versatility is likely to offer highly attractive routes to finely tune and understand immunostimulatory responses. Should their use as therapeutics be recommended in the future, the scaffolds could be easily modified to contain degradable esters or other hydrolysable bonds that would promote decomposition to below the renal filtration threshold (ca. 30–40kDa). Increasing the response to ODN by cells of the innate immune system may aid current efforts to expand vaccine supplies, and the reduction in the quantity of ODN required to elicit an immune response will ensure lower cytotoxicity and cost.[12]

Conclusion—We have described the synthesis of a novel poly(acrylic acid)-oligodeoxynucleotide conjugate that can control the presentation of ODN by variations in valency or polymer size. By monitoring the in vitro response of the macrophage-like RAW264.7 cell line to these polymers, we have demonstrated that increasing the valency of the ODN conjugates increases the potency of these scaffolds, for eliciting cytokine responses, that are up to 350-fold greater than that of monomeric ODN. We also show that the quality of the activation increases with increasing valency, eliciting high TNF- α and

much higher IL-6 production compared to that of the free ODN and polymers bearing a single ODN. TLR9 silencing by siRNA confirms that the immunological properties of these conjugates are mediated by TLR9. Current and future studies include the elucidation of the rules governing cytokine secretion profiles for ODN–PAA conjugates of differing sizes and valences, the role of these factors in inhibition of cytokine secretion, and detailed comparative analysis of intracellular concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Gel permeation chromatography of polymers and ODN samples in PBS pH 7.0, monitored at a wavelength of 260nm. Samples are ODN 1668 (5μM DNA) (open circles), **118K Biotin** (black line) (0.5μM polymer), **118K-10 Biotin** (light grey crosses) (5μM DNA and 0.5μM polymer conjugate), **118K-1** (black diamonds) (5μM DNA and 5μM conjugate), and **118K-10** (closed circles) (5μM DNA and 0.5μM conjugate).

Figure 2.

Cell titer blue viability assay of RAW264.7 cells at 8 hours. Untreated cells were monitored, as well as cells treated with conjugates **118K-1** (200nM DNA), **118K-3** (200nM DNA), and **118K-10** (200nM DNA). Standard deviation calculated from the average of 3 independent measurements. A)* indicates statistically significant differences at $p = 0.014$.

Figure 3.

Flow cytometry of RAW264.7 cells exposed to conjugates **118K Biotin-1** (light grey) and **118K Biotin-10** (hashed) normalized to 100 nM ODN concentration at 37° for 20 min, 60 min, and 120 min and 4°C for 120 min. NS = No Stain. The results presented are the MFI average of the geometric mean of 10,000 cells, n=3 samples, with error bars representing standard deviation and are representative of greater than three independent trials. Statistics between time points of each condition, * p<0.001 and ** p<0.0001.

Figure 4. Microscopy of polymer conjugate subcellular localization. Confocal image (a) is a two dimensional rendering of a 32 image z-stack of RAW264.7 cells exposed to conjugate **118K Biotin-10** (20nM conjugate/ 200nM ODN) labeled by streptavidin conjugate Qdot 525 (green), endocytosed vesicles by FM4-64FX (red), and nuclei by DRAQ5 (blue) with sites of colocalization visible as yellow (white scale bar 10 μ m). Transmission electron microscopy (b) of RAW264.7 cells exposed to polymer conjugate **118K Biotin-10** complexed with 10nm streptavidin-gold particles (black scale bar 100 nm)

Figure 5.

Cytokine release at 8 hours from RAW264.7 cells by ODN 1668 (a and b) and DNA conjugates (c and d). Comparison of TNF- α (a and c) and IL-6 (b and d) release for ODN 1668 in μ M DNA and conjugates or controls by normalized concentration DNA at 16.6nM (closed bar), 100nM (crosshatched bar), and 200nM (open bar). Concentration of 118K is the PAA concentration for conjugate 118K-1 (200nM, 100nM, or 16.6nM). Error bars represent the standard deviation from the average of three independent experimental trials and 6 independent measurements. # statistically different from the 118K-10 conjugate ($p=0.02$). ## statistically different from the 118K-10 and 118K-3 conjugates ($p=0.003$). * statistically different from the 118K-10 conjugate ($p<0.0001$). ** Statistically different from conjugate 118K-10 ($p<0.0001$) and statistically different from conjugate 118K-3 ($p=0.0148$).

Figure 6.

The effect of TLR9 siRNA knockdown on stimulation of untreated, TLR9 knockdown, or mock transfected RAW264.7 cells. Figure shows the TNF- α (pg/ mL) measured by ELISA of untreated (dark grey), TLR9 knockdown (white), or mock transfected (light grey) RAW264.7 cells by ODN 1668 (2.5 μ M), conjugate 118K-10 (200 nM), and LPS (100 ng/ mL). Error bars represent the standard deviation from the average of three independent samples. ** Indicates statistically relevant difference ($p < 0.003$).

Figure 7.

ODN 1668 titration **(a)** and response to conjugate 118K-10, ODN 1668, and ODN 1668 GC **(b)** for GMCSF matured murine macrophages as determined by IL-6 ELISA 8 hours post treatment. Panel **(a)** shows the IL-6 response at 8 hours for primary macrophages at the specified concentration of ODN 1668 in μM . Panel **(b)** is the IL-6 (pg/mL) at 8 hours for conjugate 118K-10 (dark grey), ODN 1668 (light grey), and ODN 1668 GC (white). Error bars represent the standard deviation from the average of three independent samples

Synthesis of the ODN-Poly(acrylic acid) Scaffolds

Purification and Characterization

Unpurified ODN-PAA
Conjugates 50mM Phosphate
pH 8Centrifugal Separation
50K MWCO High MW
Fraction
Removed
Volume
DeterminedConcentration determined
UV-VIS Spectroscopy
at 260 nm

$$\text{Valency} = \frac{\text{moles ODN (measured)}}{\text{moles polymer (reaction)}}$$

Scheme 1.

Reagents and conditions: (a) 118K PAA, biotin hydrazide, HBTU, and TEA (all 10mg/mL) except TEA added as liquid) in DMSO; (b) 118K PAA with EDC, HOBT, and NEAM (all 10mg/mL) in 75mM MES pH 6.05 under nitrogen; (c) maleimide polymer and reduced DNA in 50mM phosphate, 13mM citrate, 5mM EDTA ~pH 6.9 under nitrogen; *a*. Size of 35nm determined by DLS; all conjugates shown to co-elute via GPC.

Table 1

Polymers Prepared

Conjugate	Maleimide # (NMR)	Number Biotin	Theoretical Valency	Actual Valency (UV)
118K	10	0	0	0
118K-1	10	0	1	0.8
118K-1.5	10	0	2	1.5*
118K-3	43	0	4.5	3
118K-10	43	0	14	10.5
118K-10GC	43	0	14	10.5
118K Biotin	10	33	0	0
118K Biotin-1	10	33	1	0.8
118K Biotin-10	48	33	14	10

* NMR characterization was used to validate this functionality and UV characterization method (Figure S1).