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(54) Title: ANTI-INFLAMMATORY AGENTS AND METHODS OF USING THE SAME

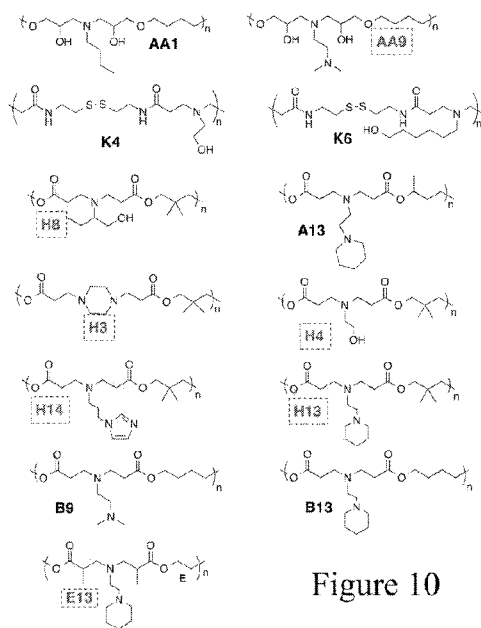


Figure 10

(57) Abstract: The present invention relates, in general, to the pro-inflammatory effects of various extracellular nucleic acids and, in particular, to methods of neutralizing the effects of such nucleic acids in a subject (e.g., a human) or in cell culture and to cationic polymers suitable for use in such methods. The invention further relates to methods of identifying anti-inflammatory cationic polymers, e.g., by screening combinatorial libraries of nucleic acid-binding polymers.



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ANTI-INFLAMMATORY AGENTS AND METHODS OF USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This patent application claims the benefit of priority of United States Provisional Patent
5 Application No. 61/810,021, filed April 9, 2013, which is incorporated herein by reference in its
entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under Grant No. R56AI093960
10 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

The present invention relates, in general, to methods of neutralizing the effects of pro-
inflammatory nucleic acids in a subject (e.g., a human) or in cells and to cationic polymers
15 suitable for use in such methods. The invention further relates to methods of identifying anti-
inflammatory cationic polymers, e.g., by screening combinatorial libraries for nucleic acid-
binding polymers.

BACKGROUND

20 Nucleic acids are released from dead and dying cells. These extracellular nucleic acids
(RNAs and DNAs) can be taken up by inflammatory cells and can activate multiple nucleic acid-
sensing Pattern Recognition Receptors (PRR) such as the Toll-Like Receptors (TLRs 3, 7, 8 and
9 in particular), which are localized in endosomes (Kawai and Akira, Nat. Immunol. 11(5):373-
84 (2010)). The inappropriate activation of these TLRs can elicit a variety of inflammatory and
25 autoimmune diseases, for example, systemic lupus erythematosus, rheumatoid arthritis and
multiple sclerosis. (Lee et al, Proc. Natl. Acad. Sci. USA 108(34):14055-60 (2011).)

It has been previously reported that certain nucleic acid-binding polymers (e.g., PAMAM-G3, CDP, HDMBR) can inhibit activation of nucleic acid-sensing PRRs, irrespective of whether they recognize ssRNA, dsRNA or hypomethylated DNA (Lee et al, Proc. Natl. Acad. Sci. USA 108(34):14055-60 (2011)). Systemic administration of such polymers can prevent
5 fatal liver injury caused by pro-inflammatory nucleic acids in a murine acute toxic shock model.

The present invention results, at least in part, from studies designed to generate a library of cationic polymers that can be screened for polymers having the ability to neutralize the effects of pro-inflammatory nucleic acids, for example, in TLR 9 activation. These studies have resulted in the identification of certain cationic polymers that can neutralize the pro-
10 inflammatory effects of any nucleic acid, regardless of the sequence, structure or chemistry of the nucleic acid.

SUMMARY OF THE INVENTION

In general, the present invention relates to identifying polymers capable of blocking the
15 pro-inflammatory effects of various extracellular nucleic acids, for example, nucleic acids released from dead and dying cells. More specifically, the invention relates to methods of neutralizing the effects of such nucleic acids in a subject (e.g., a human) and to cationic polymers suitable for use in such methods. The invention further relates to methods of identifying anti-inflammatory polymers, e.g., by screening combinatorial libraries of nucleic acid-binding
20 polymers.

In one aspect, methods of inhibiting nucleic acid-induced activation of a PRR, in particular an endosomal TLR, are provided. The methods may include contacting a cell with a cationic polymer, e.g. by adding the cationic polymer to the extracellular space or media of the cells, in an amount effective to inhibit activation of a PRR (TLR activation) by the nucleic acid.
25 The methods also include administering a cationic polymer to a subject in an amount effective to inhibit TLR activation by the nucleic acid. The administration may include systemic or localized administration of the cationic polymer compositions. The cationic polymers suitable for use in the methods are disclosed herein but are all either a poly(β -amino ester), a disulfide containing poly(β -amido amine) or a poly(β -hydroxyl amine) polymer backbone.

In another aspect, cationic polymers capable of binding to a nucleic acid and inhibiting the ability of the nucleic acid to activate a PRR, such as an endosomal TLR, are provided. Some of these cationic polymers are provided in Figure 10 wherein n is between 1 and 500, 5 to 250, 10-200, 20-150, 30-100 or any combination thereof.

5 In still another aspect, methods of screening for anti-inflammatory cationic polymers are provided. A polymer library can be screened for anti-inflammatory polymers by selecting the cationic polymers that: 1) are capable of inhibiting IL-6 production by cells in response to treatment with a nucleic acid stimulator of a TLR; 2) have minimal to no effect on the stimulation of the cell through TLR2, TLR4, or TLR5 in response to a stimulator of these TLRs;
10 3) have minimal cytotoxicity to the cells; 4) are capable of binding nucleic acids; and optionally 5) are not taken up by the cells, but remain extracellular.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a set of synthetic schemes for generation of the combinatorial nucleic acid-binding cationic polymer library. Michael Addition of primary or secondary amines to acrylate/acrylamide or epoxide ring opening of glycidyl ethers by primary or secondary amines was used to generate the polymers in the library. In generation of the libraries size was not a selection criterion. Thus n is, for example, 1 to 500.

20 Figure 2 shows the monomer structures used to generate the nucleic acid-binding polymer combinatorial library. The lettered structures (A-K and AA-CC) represent the backbone monomers. The numbered structures (1-14) represent the R side chain amine linkers, linking the monomers to form the cationic polymers. Combinatorial synthesis affords 196 polymers. The names of each of the monomers used is indicated under each structure.

25 Figure 3 is a graph and array depiction showing the results of the ethidium bromide displacement assay for some of the polymers demonstrating the ability of the cationic polymers

to bind the CpG1668 and displace the ethidium bromide. Polyethylenimine (PEI) was included as a positive control. Lighter shades indicate more displacement and a higher binding affinity.

Figure 4 is a set of arrays depicting the results of the ethidium bromide displacement assay and the CE₅₀ analysis (competitive exclusion 50) which is the amount of the polymer necessary to decrease the ethidium bromide fluorescence by 50%. The dashed lines represent three sidechain linkers showing the best ability to displace the ethidium bromide (9, 10 ad 13). Lighter shades are indicative of higher affinity of the polymers.

Figure 5A-5D is a set of bar graphs showing the ability of selected nucleic acid-binding polymers to inhibit TLR-9 activation by pro-inflammatory DNA (CpG1668) and the resulting reduction of IL-6 cytokine production at varying doses in primary dendritic cells. The effect of the polymers on TLR4 activation in response to lipopolysaccharide (LPS) was also assessed. The polymer was administered for the first 10 minutes then the CpG or LPS were added for 18 hr incubation prior to measuring IL-6 production by the cells. These polymers are selective for nucleic acids that do not inhibit synthetic, non-nucleic acid agonist LPS.

Figure 6A and 6C is a set of graphs showing the ability of selected nucleic acid-binding polymers to inhibit TLR9 activation by CpG1668 and the resulting reduction of IL-6 production at lower doses of the indicated polymers in primary dendritic cells. Figure 6B and 6D are graphs showing the effect of similar doses of the nucleic acid-binding polymers on TLR4 activation by LPS in primary dendritic cells.

Figure 7A and 7B are graphs showing the combined results from the *in vitro* assays for the polymer backbones (A-K and AA-CC in Fig. 7A) and for the monomer side chain linkers (1-14 in Fig. 7B). Polymer backbones A, B, H and K performed better than the other backbones tested. Polymer backbones C, D, E, F, and AA each produced at least one suitable polymer. Monomer side chains 1, 6, 8, 9, 13 and 14 performed better than the other monomer side chains tested. Monomer side chains 3 and 4 each produced at least one suitable polymer as well.

Figure 8 is a set of FACS analyses showing that the polymers are capable of reducing cellular uptake of a fluorescently labeled nucleic acid by cells and that the cells were cells of the macrophage/dendritic lineage (CD11b⁺ and CD11c⁺). Several of the polymers were better than PAMAM at reducing cellular uptake of the labeled CpG1668.

5 Figure 9 is a bar graph showing the percent viability of cells by an MTT assay after incubation with each of the indicated polymers. The backbone monomer of the polymer is indicated above the bar graph and the monomer side chain is indicated along the bottom of the graph. A star (*) indicates that 100% of the cells were non-viable (100% cell death). The data are presented as mean +/- standard deviation (n=4).

10 Figure 10 shows the structures of the lead candidate polymers (n is 1 to 500).

DETAILED DESCRIPTION OF THE INVENTION

The present invention results, at least in part, from studies designed to develop non-toxic, nucleic acid-binding polymers that form stable polyplexes with extracellular, pro-inflammatory nucleic acids and prevent cellular uptake, thereby inhibiting PRR activation, in particular TLR3, 7, 8, and 9 or RIG-I activation and reducing cytokine production in response to nucleic acid agonists of these receptors. Nucleic acid agonists include any nucleic acid capable of activating a PRR and inducing a cell to produce cytokines such as IL-6. Nucleic acid agonists include dsRNA, ssRNA, un- or hypo-methylated DNA or ssDNA and the agonists may be complexed with proteins. The present invention relates, at least in part, to a combinatorial library of nucleic acid-binding cationic polymers and to methods of identifying cationic polymers suitable for use as anti-inflammatory therapeutics in a subject (e.g., a mammal, preferably, a human) comprising screening the library for polymers that neutralize or inhibit the effects of pro-inflammatory nucleic-acids. The invention further relates to anti-inflammatory polymers so identified and to methods of using same.

25 A combinatorial library of 196 polymers (140 poly(β -amino ester)s, 14 disulfide containing poly(β -amido amine)s and 42 poly(β -hydroxyl amine)s) were synthesized from the reactions of primary or secondary amines with either bisacrylates, bisacrylamides or

diglycidylethers (Fig. 1) (poly(β -amino ester)s: Akinc et al, *Bioconjugate Chemistry* 14(5): 979-988 (2003); poly(β -hydroxyl amine)s: Barua et al, *Molecular Pharmaceutics* 6(1): 86-97 (2009); and poly(β -amido amine)s: Lin et al, *Bioconjugate Chemistry* 18(1): 138-145 (2007)). These cationic polymer classes have been studied for the purpose of non-viral gene delivery (e.g., siRNA) (Pack et al, *Nat. Rev. Drug Discov.* 4:581-593 (2005)), however, they have not been studied in other therapeutic areas, such as inflammation and thrombosis. While these polymers were developed to bind to and deliver nucleic acids into the nuclei of cells, they can be chemically tuned (e.g., by the selection of backbone monomer functionalities) to prevent cellular uptake and improve anti-inflammatory function.

As detailed in the Example that follows, these nucleic acid-binding polymers were tested for their ability to inhibit TLR-9 activation in the presence of pro-inflammatory ssDNA. Primary bone marrow-derived plasmacytoid dendritic cells were incubated with varying doses of nucleic acid-binding polymers and a subsequent dose of a known TLR-9 agonist. Functional polymers were selected based on their ability to decrease IL-6 production after an overnight incubation without suppressing cytokine production in the presence of a non-nucleic acid TLR agonist, LPS (Fig. 5-6). This demonstrated the selectivity of these cationic polymers for nucleic acids.

Potential candidates were subjected to further screening for cytotoxicity, cellular uptake, and ability to bind nucleic acids as described in the example and shown in Figures 3, 4, 8 and 9. The polymers suitably have low or no cytotoxicity. At doses of the polymers provided herein required to inhibit PRR activation by a nucleic acid agonist, the cationic polymers provided herein are not cytotoxic or have low cytotoxicity as compared to untreated cells. Low cytotoxicity indicates that cell viability in cells treated with the polymer is reduced by less than 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45% or 50% as compared to untreated control cells. Suitably, the cationic polymers provided herein do not allow or inhibit cellular uptake of the nucleic acid agonists. Without being limited by theory, we hypothesize that the cationic polymers provided herein work at least partially by inhibiting cellular uptake of the nucleic acid agonists of the PRRs and thus inhibit interaction of the nucleic acid agonists with the receptors. The cationic polymers may inhibit uptake of the nucleic acid by 10%, 20%, 30%, 40%, 50% or even 60% or more as compared to control cells provided a distinct polymer or no polymer at all.

The present invention relates, in one embodiment, to methods of inhibiting nucleic acid-induced activation of PRRs, such as endosomal TLRs (e.g., TLR 9). The methods include

contacting a cell with a cationic polymer (e.g., by adding the polymer to the extracellular space or media) or administering to a subject (e.g., a human) in need thereof the cationic polymers described herein. The cationic polymer is capable of binding nucleic acids responsible for induction of PRR (TLR) activation in an amount and under conditions such that inhibition of that activation is effected. Advantageously, the agent binds the nucleic acids in a manner that is independent of the nucleotide sequence, the chemistry (e.g., DNA or RNA, with or without base or sugar modifications) and/or the structure (e.g., double-stranded or single-stranded, complexed or uncomplexed with, for example, a protein) of the nucleic acids responsible for inducing nucleic acid receptor (TLR) activation. The present method can be used to treat inflammatory and/or autoimmune responses resulting from inappropriate activation of nucleic acid receptors on or in cells. Administration or addition of the cationic polymers inhibits activation of the nucleic acid receptor by 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or more in a dose-dependent manner such that addition of small amounts of the cationic polymer are not or only slightly capable of inhibiting receptor activation and addition of higher amounts of the cationic polymers results in additional inhibition up to full inhibition of activation of the receptor by the nucleic acid. The percentage inhibition of the receptor may refer to the percentage inhibition or reduction in cytokine production (e.g. IL-6) in response to the nucleic acid agonist in combination with one or more of the cationic polymers as compared to cells treated with the nucleic acid agonist alone or the nucleic acid agonist and an irrelevant polymer.

Nucleic acid-binding polymers of the invention include pharmaceutically acceptable cationic polymers that can bind pro-inflammatory nucleic acids in, for example, biologic fluids, prevent cellular uptake and thereby inhibit TLR activation. Such cationic polymers include poly(β -amino ester)s, disulfide containing poly(β -amido amine)s and poly(β -hydroxyl amine)s. Preferred polymers include those in Fig. 10, particularly preferred are AA9, H3, H4, H8, H13 and H14 where "n" is, for example, 1 to 500, preferably, 5 to 250, more preferably, 10-200, 20-150 or 30-100. Other suitable polymers include A1, A2, A6, A9, A13, A14, B5, B6, B8, B9, B13, E13, F6, F8, F9, H2, H3, H4, H6, H7, H8, H9, H13, H14, I1, I13, K4, K6, K9, K14, AA1, AA9, and BB1. For each of the listed polymers the backbone is the structure listed as A-K or AA-CC as shown in Figure 2 and the monomer side chain has the structure indicated as 1-14 in Figure 2. The polymers are made from the monomers shown in Figure 2 using the reactions shown in Figure 1 to generate the polymers listed. From the results in the Example the most

suitable backbone polymers were A, B, H, K and AA and the most suitable side chain monomer linkers were 1, 6, 8, 9, 13 and 14. Cationic polymers of the invention include biodegradable and non-biodegradable polymers and blends or copolymers thereof.

Advantageously, the binding affinity of a nucleic acid-binding cationic polymer of the invention for a nucleic acid, expressed in terms of K_d , is in the pM to mM range, preferably, less than or equal to 50 nM; expressed in terms of binding constant (K), the binding affinity is advantageously equal to or greater than $10^5 M^{-1}$, preferably, $10^5 M^{-1}$ to $10^8 M^{-1}$, more preferably, equal to or greater than $10^6 M^{-1}$. Thus, the binding affinity of the sequence-independent nucleic acid-binding cationic polymers can be, for example, about $1 \times 10^5 M^{-1}$, $5 \times 10^5 M^{-1}$, $1 \times 10^6 M^{-1}$, $5 \times 10^6 M^{-1}$, $1 \times 10^7 M^{-1}$, $5 \times 10^7 M^{-1}$; or about 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M, 100 μ M. “ K ” and “ K_d ” can be determined by methods known in the art, including Isothermal Calorimetry (ITC), Forster Resonance Energy Transfer (FRET), surface plasmon resonance or a real time binding assay such as Biacore.

Preferred nucleic acid-binding cationic polymers of the invention simultaneously limit the activation of multiple nucleic acid binding PRRs (endosomal TLRs, e.g., TLR3, TLR7, TLR8 and TLR9 and possibly cytosolic nucleic acid sensors such as RIG-I) by binding to a wide array of different nucleic acids including ssRNA, ssDNA, dsRNA and dsDNA and of which may be presented in a complex with protein such as viral proteins, histones, HMGB1 or RIG-I. Suitably the nucleic acid-binding polymers do not inhibit activation of non-nucleic acid binding TLRs such as TLR 2, TLR4, TLR5, or TLR6. For example, the cationic polymers do not inhibit activation by LPS, lipoproteins, or flagellin. The cationic polymers are not taken up by the cells and are not cytotoxic.

As indicated above, the present invention provides a method of controlling (inhibiting or preventing) autoimmune and/or inflammatory responses associated with activation of PRRs by nucleic acids (e.g., endosomal TLRs, such as TLR9). Such responses play a role in the pathogenesis of diseases/disorders that are associated with presence in the circulation of the subject of free nucleic acids, either pathogen-derived (e.g., viral- or bacterial-derived) nucleic acids or nucleic acids released from dead or damaged host cells. Specific diseases/disorders that can be treated using nucleic acid-binding polymers of the invention include infectious diseases, cardiovascular disease, cancer, bacterial sepsis, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, inflammatory bowel disease, COPD, obesity, psoriasis, atherosclerosis,

diabetes, wound healing, burns, infectious diseases, reperfusion injury, renal failure/dialysis, organ transplantation, neurodegenerative disease and traumatic brain injury. (See also PCT/US2010/002516, filed September 16, 2010.)

The cationic polymers can also be used in combination with other treatments. The cationic polymers may be used in conjunction with another therapeutic, such as a cancer therapeutic, known to result in a robust inflammatory response by releasing nucleic acids. Such treatments may be treatments known to induce cell death or nucleic acid based inflammation. In one embodiment, the cationic polymers are administered to cells or a subject which previously received or were exposed to a nucleic acid-based pharmaceutical composition, such as an siRNA, DNA vaccine or aptamer based therapy. The polymers described herein may be useful to limit inflammatory side effects associated with administration of such therapeutics.

Another application of nucleic acid-binding polymers described herein is to counteract the effects of DNA, RNA or polyphosphate molecules that are released from cells and subsequently induce thrombosis (Kannemeier et al, Proc. Natl. Acad. Sci. 104:6388-6393 (2007); Fuchs et al, Proc. Natl. Acad. Sci. Published Online before Print August 23, 2010). It has been observed that RNA and DNA molecules can activate the coagulation pathway as well as platelets and thereby engender blood clotting (Kannemeier et al, Proc. Natl. Acad. Sci. 104:6388-6393 (2007); Fuchs et al, Proc. Natl. Acad. Sci. Published Online before Print August 23, 2010). Since nucleic acid-binding cationic polymers described herein can bind RNA and DNA molecules and shield them from other potential binding partners, such agents can be employed to inhibit the ability of DNA and RNA molecules to bind and activate coagulation factors and platelets. In so doing, these RNA/DNA-binding polymers can be utilized to limit nucleic acid-induced pathological blood coagulation. Thus, nucleic acid-binding cationic polymers described herein represent novel entities for preventing the induction and progression of a variety of thrombotic disorders, including myocardial infarction, stroke and deep vein thrombosis.

For use in the methods described herein, cells may be contacted with the polymers directly or indirectly *in vivo*, *in vitro*, or *ex vivo*. Contacting encompasses administration to a cell, tissue, mammal, subject, patient, or human. Further, contacting a cell includes adding the polymers to a cell culture, such as by including or adding the polymer to the media in which the cell is incubating or providing the polymer to the extracellular space. Other suitable methods

may include introducing or administering the polymers described herein to a cell, tissue, mammal, or patient using appropriate procedures and routes of administration as defined below.

The nucleic acid-binding polymers of the invention, or pharmaceutically acceptable salts thereof, can be administered to the subject via any route such that effective levels are achieved in, for example, the bloodstream. The compounds described herein may be administered by any means including, but not limited to, oral, topical, intranasal, intraperitoneal, parenteral, intravenous, intramuscular, subcutaneous, intrathecal, transcutaneous, transdermal, nasopharyngeal, or transmucosal absorption. Thus the compounds may be formulated as an ingestible, injectable, topical or suppository formulation. The compounds may also be delivered with in a liposomal or time-release vehicle. The nucleic acid-binding polymer can also be administered, for example, directly to a target site, for example, directly to a joint when arthritis is the disease to be treated. Advantageously, the nucleic acid-binding polymer is administered as soon as clinical symptoms appear and administration is repeated as needed.

Administration of the compounds to a subject in accordance with the invention appears to exhibit beneficial effects in a dose-dependent manner. The optimum dosing regimen will depend, for example, on the nucleic acid-binding polymer, the subject, the condition being treated and the effect sought. Thus, within broad limits, administration of larger quantities of the compounds is expected to achieve increased beneficial biological effects than administration of a smaller amount. Moreover, efficacy is also contemplated at dosages below the level at which toxicity is seen.

It will be appreciated that the specific dosage administered in any given case will be adjusted in accordance with the compound or compounds being administered, the disease to be treated or inhibited, the condition of the subject, and other relevant medical factors that may modify the activity of the compound or the response of the subject, as is well known by those skilled in the art. For example, the specific dose for a particular subject depends on age, body weight, general state of health, diet, the timing and mode of administration, the rate of excretion, medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the compound of the invention and of a known agent, such as by means of an appropriate conventional pharmacological or prophylactic protocol.

The maximal dosage for a subject is the highest dosage that does not cause undesirable or intolerable side effects. The number of variables in regard to an individual prophylactic or treatment regimen is large, and a considerable range of doses is expected. The route of administration will also impact the dosage requirements. It is anticipated that dosages of the compound will reduce symptoms of the condition at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% compared to pre-treatment symptoms or symptoms is left untreated. It is specifically contemplated that pharmaceutical preparations and compositions may palliate or alleviate symptoms of the disease without providing a cure, or, in some embodiments, may be used to reverse the disease or disorder, such as an autoimmune or inflammatory disease.

Suitable effective dosage amounts for administering the compounds may be determined by those of skill in the art, but typically range from about 1 microgram to about 500,000 micrograms per kilogram of body weight weekly, although they are typically about 100 milligrams or less per kilogram of body weight weekly. The effective dosage amounts described herein refer to total amounts administered, that is, if more than one compound is administered, the effective dosage amounts correspond to the total amount administered. The compound can be administered as a single dose or as divided doses. For example, the composition may be administered two or more times separated by 4 hours, 6 hours, 8 hours, 12 hours, a day, two days, three days, four days, one week, two weeks, or by three or more weeks. If added to cellular media, the cationic polymers may be added such that the concentration of the polymer is between 10nM and 10mM, 50nM and 5mM, 100nM and 3mM, or 500nM and 2mM.

The nucleic acid-binding cationic polymers, or pharmaceutically acceptable salts thereof, can be formulated with a carrier, diluent or excipient to yield a pharmaceutical composition. The compounds may be used to make pharmaceutical compositions. Pharmaceutical compositions comprising the compound of formula (I) or any of the compounds described above and a pharmaceutically acceptable carrier are provided. A pharmaceutically acceptable carrier is any carrier suitable for *in vivo* administration. Examples of pharmaceutically acceptable carriers suitable for use in the composition include, but are not limited to, water, buffered solutions, glucose solutions, oil-based or bacterial culture fluids. Additional components of the compositions may suitably include, for example, excipients such as stabilizers, preservatives, diluents, emulsifiers and lubricants. Examples of pharmaceutically acceptable carriers or diluents include stabilizers such as carbohydrates (e.g., sorbitol, mannitol, starch, sucrose,

glucose, dextran), proteins such as albumin or casein, protein-containing agents such as bovine serum or skimmed milk and buffers (e.g., phosphate buffer). Especially when such stabilizers are added to the compositions, the composition is suitable for freeze-drying or spray-drying. The composition may also be emulsified.

5 The precise nature of the compositions of the invention will depend, at least in part, on the nature of the nucleic acid-binding polymer and the route of administration. It will be appreciated that the treatment methods of the present invention are useful in the fields of both human medicine and veterinary medicine. Thus, the patient (subject) to be treated can be a mammal, preferably a human. For veterinary purposes the subject can be, for example, a farm
10 animal such as a cow, pig, horse, goat or sheep, or a companion animal such as a dog or a cat.

The cationic polymers described herein may be administered in combination with each other or in combination with other therapeutics such as an antimicrobial, cancer therapeutic, nucleic acid based therapeutic or inflammatory mediator. The compositions may be administered in any order, at the same time or as part of a unitary composition. The two
15 therapeutics may be administered such that one is administered before the other with a difference in administration time of 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, 1 day, 2 days, 4 days, 7 days, 2 weeks, 4 weeks or more.

An effective amount or a therapeutically effective amount as used herein means the amount of a composition that, when administered to a subject for treating a state, disorder or
20 condition is sufficient to effect a treatment. The therapeutically effective amount will vary depending on the compound, formulation or composition, the disease and its severity and the age, weight, physical condition and responsiveness of the subject to be treated as described for dosing above.

The invention also relates to methods of identifying nucleic acid-binding polymers
25 suitable for use in the above-described methods. In one embodiment, endosomal TLR-containing cells, preferably, mammalian cells (e.g., mammalian plasmacytoid dendritic cells), are incubated with a first PRR agonist such as an endosomal TLR agonist (e.g., CpG DNA or single or double stranded RNA or nucleic acid-containing particles) in the presence and absence of a test agent (e.g., a cationic polymer selected from the combinatorial library described above and
30 in the Example that follows). Following incubation, a culture supernatant sample can be taken and analyzed for the presence of a product of an intracellular signaling event initiated by

activation of the nucleic acid responsive receptor (TLR9), for example, one or more cytokines (e.g., IL-6). These steps can be repeated with a PRR agonist or an endosomal TLR agonist having a sequence, chemistry and/or structure different from that of the first agonist, e.g. dsRNA. A test agent that inhibits activation of the nucleic acid responsive receptor, preferably, in a manner independent of the sequence, chemistry and/or structure of the nucleic acid agonist used (that inhibition of activation being evidenced by inhibition of production of a product of an intracellular signaling event initiated by PRR activation (e.g., cytokine production) (e.g., in a dose dependent manner)) can then be tested *in vivo*, for example, in mice, to further assess its suitability for use in the methods described herein.

Suitably the polymers are also tested for the inability to block activation and cytokine production by cells in response to non-nucleic acid binding PRRs (TLRs) such as LPS activation of TLR4; Pam3CSK4 activation of TLR2; endogenous DAMP or heparan sulfate activation of TLR4. The polymers should also be tested for cytotoxicity to cells after incubation and for lack of toxicity when administered to subjects such as mice. Cytotoxic or toxic polymers should not be selected whereas those having no or little toxicity may be selected for further evaluation. Suitably the polymers are also selected for the ability to bind to the nucleic acids with high affinity and to not be taken up by cells into an intracellular space.

Certain aspects of the invention are described in greater detail in the non-limiting Example that follows. (See also Oney et al, Nat. Med. 15(10):1224-8 (2009) and PCT/US2010/002516, filed September 16, 2010.)

The present disclosure is not limited to the specific details of construction, arrangement of components, or method steps set forth herein. The compositions and methods disclosed herein are capable of being made, practiced, used, carried out and/or formed in various ways that will be apparent to one of skill in the art in light of the disclosure that follows. The phraseology and terminology used herein is for the purpose of description only and should not be regarded as limiting to the scope of the claims. Ordinal indicators, such as first, second, and third, as used in the description and the claims to refer to various structures or method steps, are not meant to be construed to indicate any specific structures or steps, or any particular order or configuration to such structures or steps. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to

facilitate the disclosure and does not imply any limitation on the scope of the disclosure unless otherwise claimed. No language in the specification, and no structures shown in the drawings, should be construed as indicating that any non-claimed element is essential to the practice of the disclosed subject matter. The use herein of the terms “including,” “comprising,” or “having,”
5 and variations thereof, is meant to encompass the elements listed thereafter and equivalents thereof, as well as additional elements. Embodiments recited as “including,” “comprising,” or “having” certain elements are also contemplated as “consisting essentially of” and “consisting of” those certain elements.

Recitation of ranges of values herein are merely intended to serve as a shorthand method
10 of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and
15 all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure. Use of the word “about” to describe a particular recited amount or range of amounts is meant to indicate that values very near to the recited amount are included in that amount, such as values that could or naturally would be accounted for due to manufacturing tolerances, instrument and human
20 error in forming measurements, and the like. All percentages referring to amounts are by weight unless indicated otherwise.

No admission is made that any reference, including any non-patent or patent document cited in this specification, constitutes prior art. In particular, it will be understood that, unless otherwise stated, reference to any document herein does not constitute an admission that any of
25 these documents forms part of the common general knowledge in the art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein. All references cited herein are fully incorporated by reference, unless explicitly indicated otherwise. The present disclosure shall control in the event there are any disparities
30 between any definitions and/or description found in the cited references.

The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

5 Reactions employed to generate a combinatorial library for functional screening is set forth in Fig. 1 and the monomer building blocks used to synthesize the polymer library are set forth in Fig. 2. Amine monomers 1-14 were reacted with backbone monomers (A-K, AA-CC) in 1:1 stoichiometry at 1.6 M in DMSO for 5 days at 56°C in multi-well polypropylene plates. The library consists of 3 main polymer classes, namely, poly(β -amino ester)s, disulfide containing
10 poly(β -amido amine)s and poly(β -hydroxyl amine)s. The monomer building blocks were selected to include multiple functionalities in both the backbone and side-chains of the polymer to enhance biodegradability and bioreducibility and to lower toxicity and reduce cellular uptake (Fig. 2). The poly(β -amino ester)s, and poly(β -amido amine)s, were synthesized via Michael-type additions of amines to bisacrylates or bisacrylamides to generate (poly(β -amino ester)s (see
15 Akinc et al, *Bioconjugate Chemistry* 14(5): 979-988 (2003)); and poly(β -amido amine)s (see Lin et al, *Bioconjugate Chemistry* 18(1): 138-145 (2007)). The poly(β -hydroxyl amine)s were synthesized via epoxide ring opening of diglycidyl ethers by either primary or secondary amines (see Barua et al, *Molecular Pharmaceutics* 6(1): 86-97 (2009)). These reactions are robust, efficient and offer a modular strategy by which a large number of chemical functionalities can be
20 introduced onto a polymeric backbone in a combinatorial fashion.

The 196 polymers made using the methods above were then screened for their ability to bind to CpG1668 oligodeoxynucleotides (ODN) using an ethidium bromide displacement assay as described by (Tse and Boger (2005) *Current Protocols in Nucleic Acid Chemistry*, 20:8.5.1–8.5.11.). Briefly, CpG oligodeoxynucleotides (CpG1668 ODN) were incubated with ethidium
25 bromide to allow intercalation ($[P]/[EtBr] = 4/1$) in a 96 well plate. The 196 polymers of the library were added to the CpG:EtBr complexes. The fluorescence of the complexes was measured using a 96 well plate reader (excitation wavelength = 540 nm; emission wavelength = 590 nm). The relative fluorescence at various nucleic acid to polymer ratios was calculated as relative fluorescence = $(F_{590} - F_{background})/F_0$. The results are shown in Figure 3. The graph shows
30 a representative set of polymers. Polymer A4 (diamonds) was not able to bind the CpG ODN

and did not result in decreased fluorescence of the CpG:EtBr complex. In contrast, polymers B9 (upright triangles), A13 (downward triangles), B13 (squares) and positive control PEI (polyethylenimine) were able to bind the CpG ODN. As shown in Figure 4, the affinity of the polymers for CpG ODN was evaluated by nonlinear regression analysis of the binding assay (shown in the left panel) and the CE_{50} (the amount of polymer required to decrease EtBr fluorescence by 50%) was calculated for each of the polymers as shown in the panel on the right. As noted by the dashed line, three of the side chains (9, 10 and 13) show high affinity for CpG ODN and compete with EtBr effectively.

The 196 nucleic acid-binding polymers were also tested for their ability to inhibit TLR-9 activation in the presence of a pro-inflammatory ssDNA (CpG). Primary bone marrow-derived plasmacytoid dendritic cells were incubated with varying doses of the nucleic acid-binding polymers for 10 minutes and a subsequent dose of a known TLR-9 agonist, CpG1668 was added for 18 hours. Functional polymers were selected based on their ability to decrease IL-6 production in response to the CpG ODN after a period of 18 hours without suppressing IL-6 production in the presence of LPS - a synthetic, non-nucleic acid TLR4 agonist (Fig. 5). This assay demonstrated the selectivity of these cationic polymers for nucleic acids. Further dose curves for the ability of the polymers to inhibit IL-6 production by the cells in response to CpG, but not inhibit the response to LPS are shown in Fig. 6A-D. Fig. 7A and Fig. 7B compile the data from these experiments into a structural analysis for the backbone and side chain monomers, respectively.

The polymer candidates were also tested for cellular uptake using an Alexa488-CpG uptake FACS assay (Lee et al, Proc. Natl. Acad. Sci. USA 108(34):14055-14060 (2011)). Briefly, Alexa488-CpG was incubated with the indicated polymers and added to RAW264.7 cells. The uptake of the fluorescently labeled CpG was assayed by FACS analysis and a representative set of FACS plots for polymer A13, B9, B13 and E13 are shown in Fig. 8. Each of the polymers shown was able to inhibit uptake of CpG by the RAW264.7 cells. Below each uptake FACS plot is a separate FACS analysis demonstrating that the cells in the FACS were $CD11b^+$, $CD11C^+$ cells indicating the cells are macrophage lineage cells.

Potential candidates (62 polymers) were then subjected to further screening for cytotoxicity in a murine macrophage cell line, RAW264.7. The polymers were added to the cells at a concentration of 0.5 mg/mL for 24 hours prior to being washed with PBS and the MTT assay

reagent added to the cell for 2 hours. An MTT assay was performed to assess the effect of the polymers on cell viability. The results are shown in Figure 9. The percentage of viable cells is by comparison of the cells incubated with the polymer as compared to similarly treated cells incubated in the absence of polymer. Many of the polymers tested had little or no effect on cellular viability.

The refined candidates consist of those displaying a high inhibition of TLR-9 activation in primary cells, low cytotoxicity as determined by MTT assays, low inhibition of LPS mediated activation and low cellular uptake of Alexa488-CpG. The lead candidate polymers are shown in Figure 10.

CLAIMS

We claim:

1. A method of inhibiting nucleic acid-induced activation of a pattern recognition receptor comprising contacting a cell with a cationic polymer or adding the cationic polymer to the media of cells in an amount effective to inhibit activation of the pattern recognition receptor by the nucleic acid, wherein the cationic polymer is a poly(β -amino ester), disulfide containing poly(β -amido amine) or poly(β -hydroxyl amine).
2. A method of inhibiting nucleic acid-induced activation of a pattern recognition receptor comprising administering a cationic polymer to a subject in an amount effective to inhibit TLR activation by the nucleic acid, wherein the cationic polymer is a poly(β -amino ester), disulfide containing poly(β -amido amine) or poly(β -hydroxyl amine).
3. The method of any one of the preceding claims, wherein the pattern recognition receptor is a toll-like receptor (TLR).
4. The method of any one of the preceding claims, wherein the cationic polymers do not block activation of TLR4 or TLR2.
5. The method of any one of the preceding claims, wherein the cationic polymer has low or no cytotoxicity.
6. The method of any one of the preceding claims, wherein the nucleic acid-induced activation of the TLR is inhibited by at least 50% as measured by IL-6 production in response to the nucleic acid.
7. The method of any one of the preceding claims, wherein the cationic polymer is effective at concentrations of 100 nM to 3 mM.
8. The method of any one of the preceding claims, wherein the nucleic acid is ssRNA, dsRNA, ssDNA, dsDNA or protein-nucleic acid complexes.
9. The method of any one of the preceding claims, wherein the pattern recognition receptor is TLR3, TLR7, TLR8 or TLR9.
10. The method of any one of the preceding claims, wherein the polymer backbone monomer is selected from one of the following structures: A, B, H, K, and AA.

11. The method of claim 10, wherein the side chain monomers are selected from one of: 1, 6, 8, 9, 13 and 14.
12. The method of any one of claims 1-11, wherein the cationic polymer is selected from A1, A2, A6, A9, A13, A14, B5, B6, B8, B9, B13, E13, F6, F8, F9, H2, H3, H4, H6, H7, H8, H9, H13, H14, I1, I13, K4, K6, K9, K14, AA1, AA9, and BB1.
13. The method of claim 12, wherein the cationic polymer is selected from A13, AA1, AA9, B9, B13, E13, H3, H4, H8, H13, H14, K4 and K6.
14. The method of any one of the preceding claims, wherein the cells or the subject were previously exposed to a nucleic acid-based pharmaceutical composition.
15. The method of any one of claims 2-14, wherein the subject received or is concurrently receiving a treatment inducing cell death or nucleic acid-based inflammation.
16. The method of claim 15, wherein the subject is being treated for cancer, toxic shock, bacterial sepsis, wound healing, burns, infectious diseases, reperfusion injury, renal failure/dialysis, organ transplantation, neurodegenerative disease and traumatic brain injury.
17. The method of any one of claims 2-14, wherein the subject has an autoimmune or inflammatory disease.
18. The method of claim 17, wherein the disease is selected from systemic lupus erythematosus (SLE), rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, chronic obstructive pulmonary disease (COPD), obesity, cardiovascular disease, atherosclerosis, diabetes and psoriasis.
19. A method of screening for anti-inflammatory cationic polymers comprising generating a polymer library, selecting the cationic polymers capable of inhibiting IL-6 production by cells in response to treatment with a nucleic acid stimulator of a TLR, selecting the cationic polymers that have minimal to no effect on the stimulation of the cell through TLR2, TLR4, or TLR5, selecting the cationic polymers having minimal cytotoxicity and selecting the cationic polymers capable of binding nucleic acids.
20. The method of claim 19, further comprising selecting the cationic polymers that are not taken up by the cells and remain extracellular.

21. A cationic polymer capable of binding to a nucleic acid and blocking the ability of the nucleic acid to activate at least one of TLR3, TLR7 and TLR9, the cationic polymer selected from A13, AA1, AA9, B9, B13, E13, H3, H4, H8, H13, H14, K4 and K6.
22. The cationic polymer of claim 20, wherein the cationic polymer is AA9.
- 5 23. The cationic polymer of claim 20, wherein the cationic polymer is E13.
24. The cationic polymer of claim 20, wherein the cationic polymer is H3.
25. The cationic polymer of claim 20, wherein the cationic polymer is H4.
26. The cationic polymer of claim 20, wherein the cationic polymer is H8.
27. The cationic polymer of claim 20, wherein the cationic polymer is H13.
- 10 28. The cationic polymer of claim 20, wherein the cationic polymer is H14.
29. A pharmaceutical composition comprising the cationic polymer of any one of claims 21-28 and a pharmaceutically acceptable carrier.

Figure 1

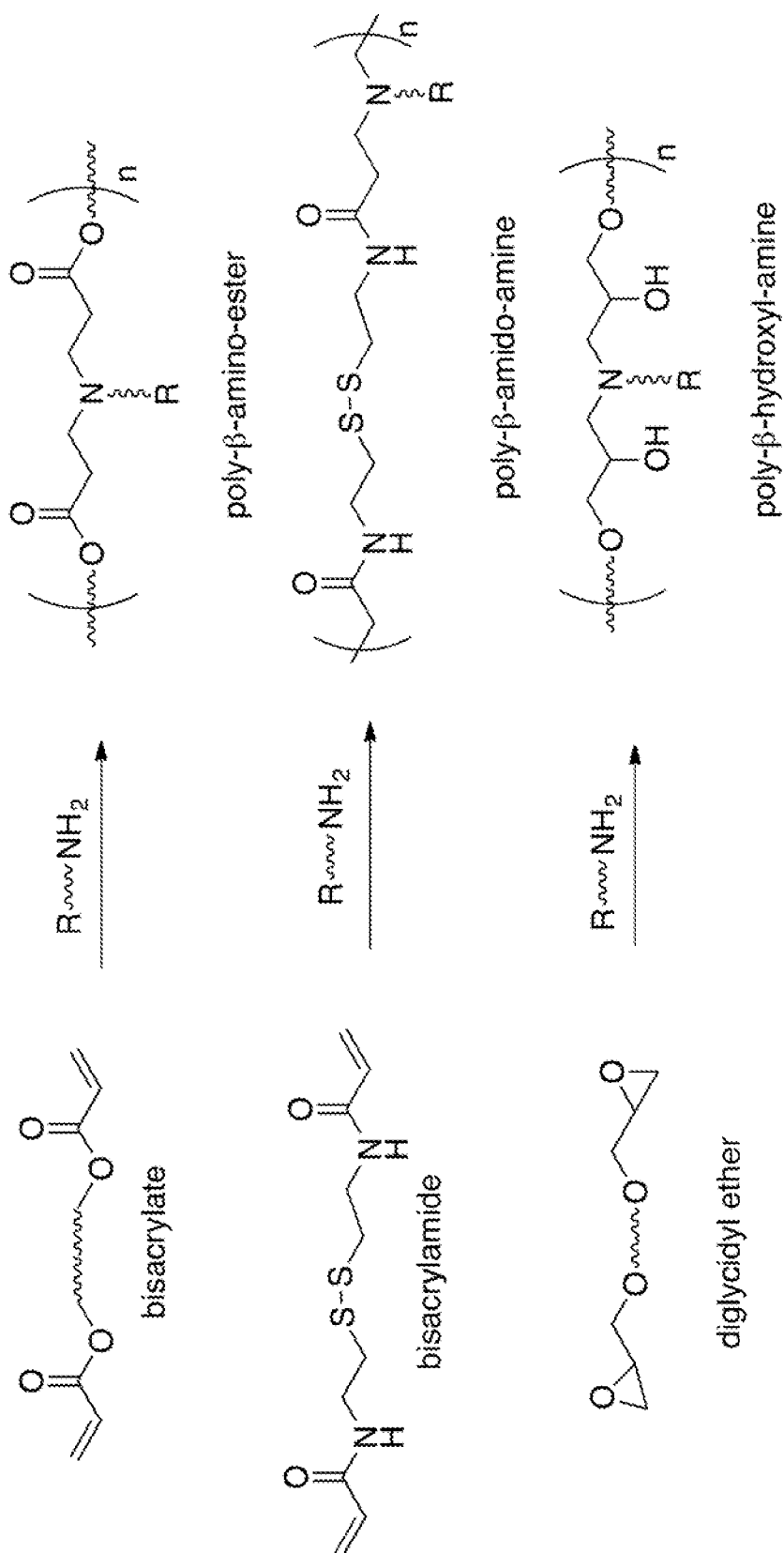


Figure 2

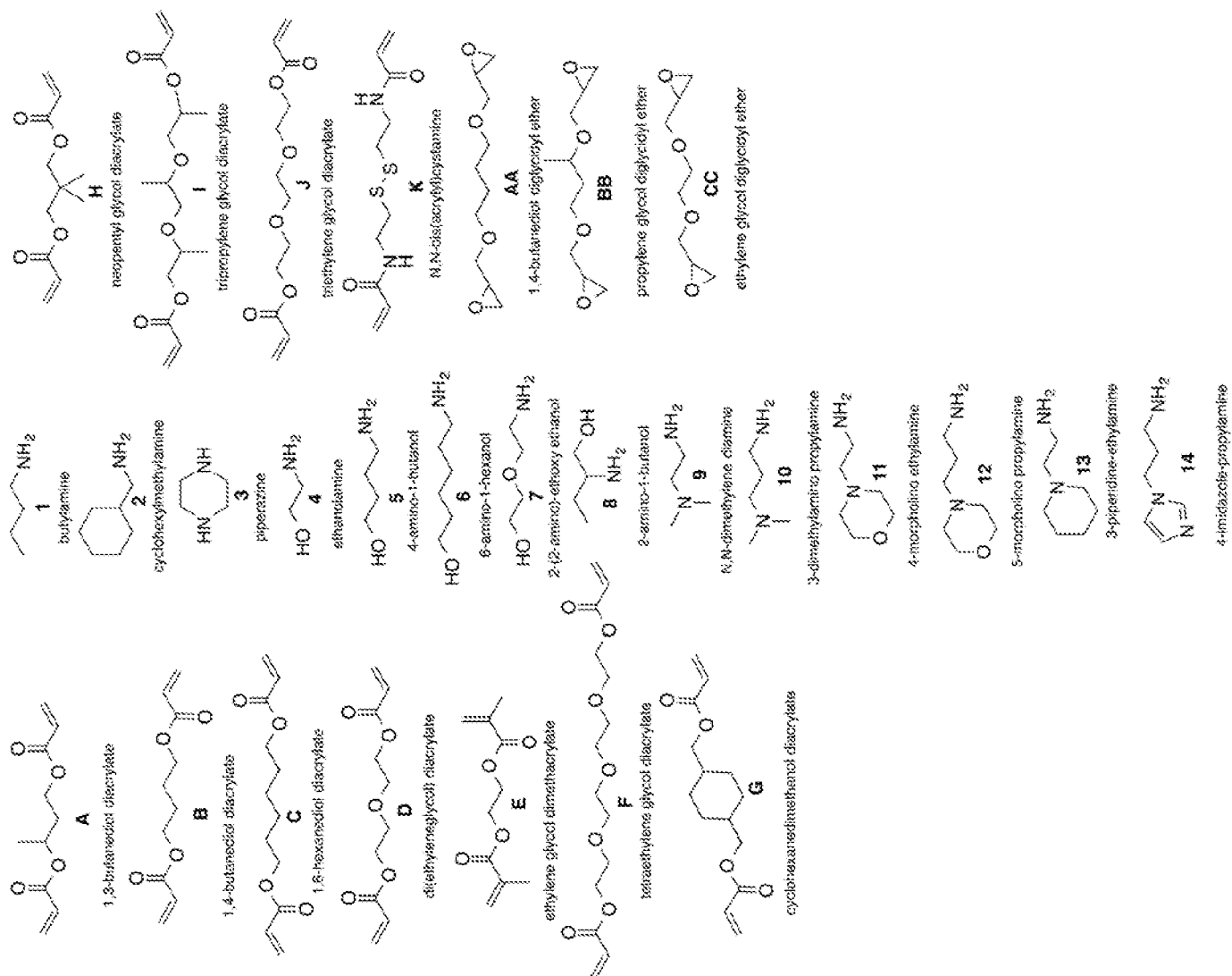


Figure 3

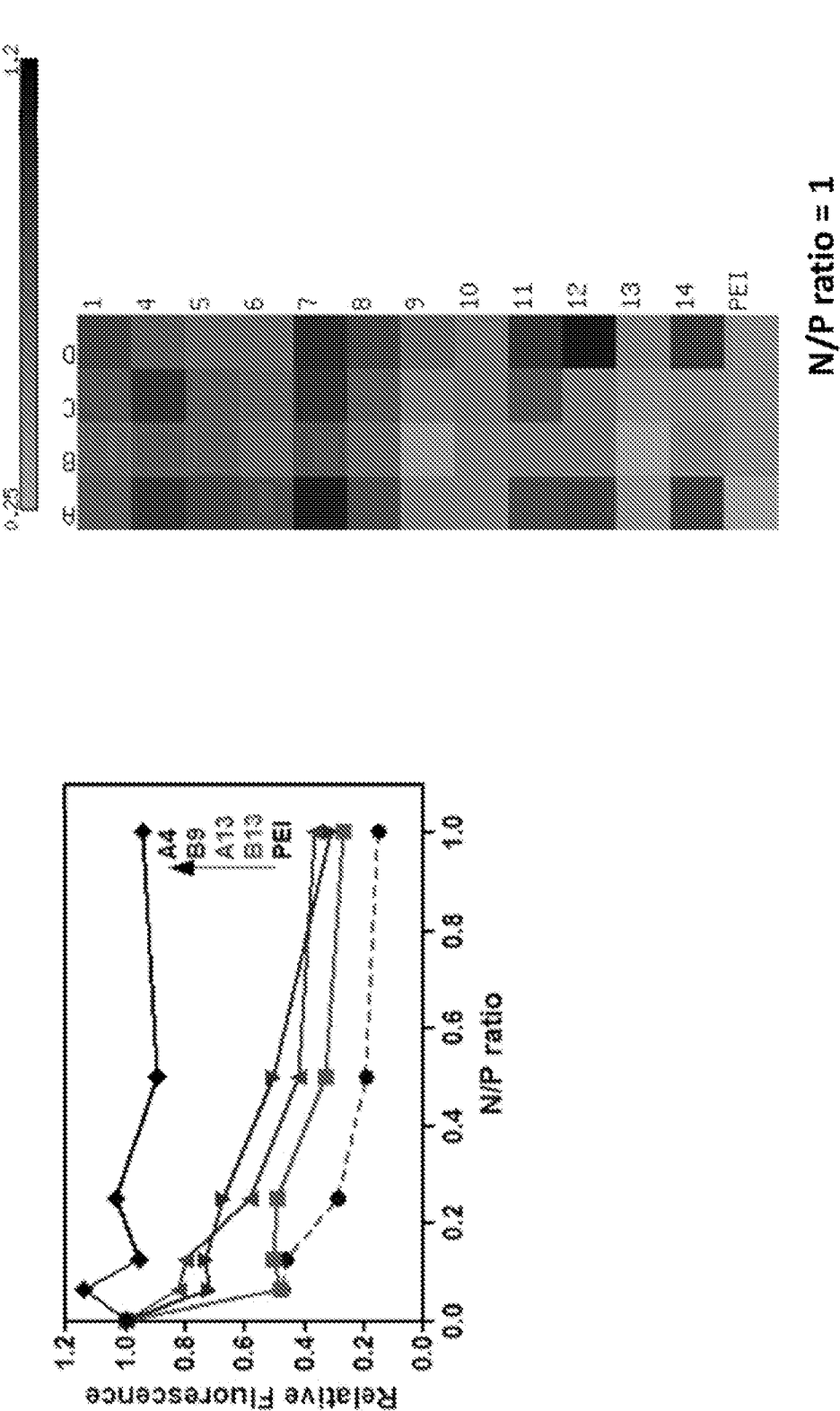


Figure 4

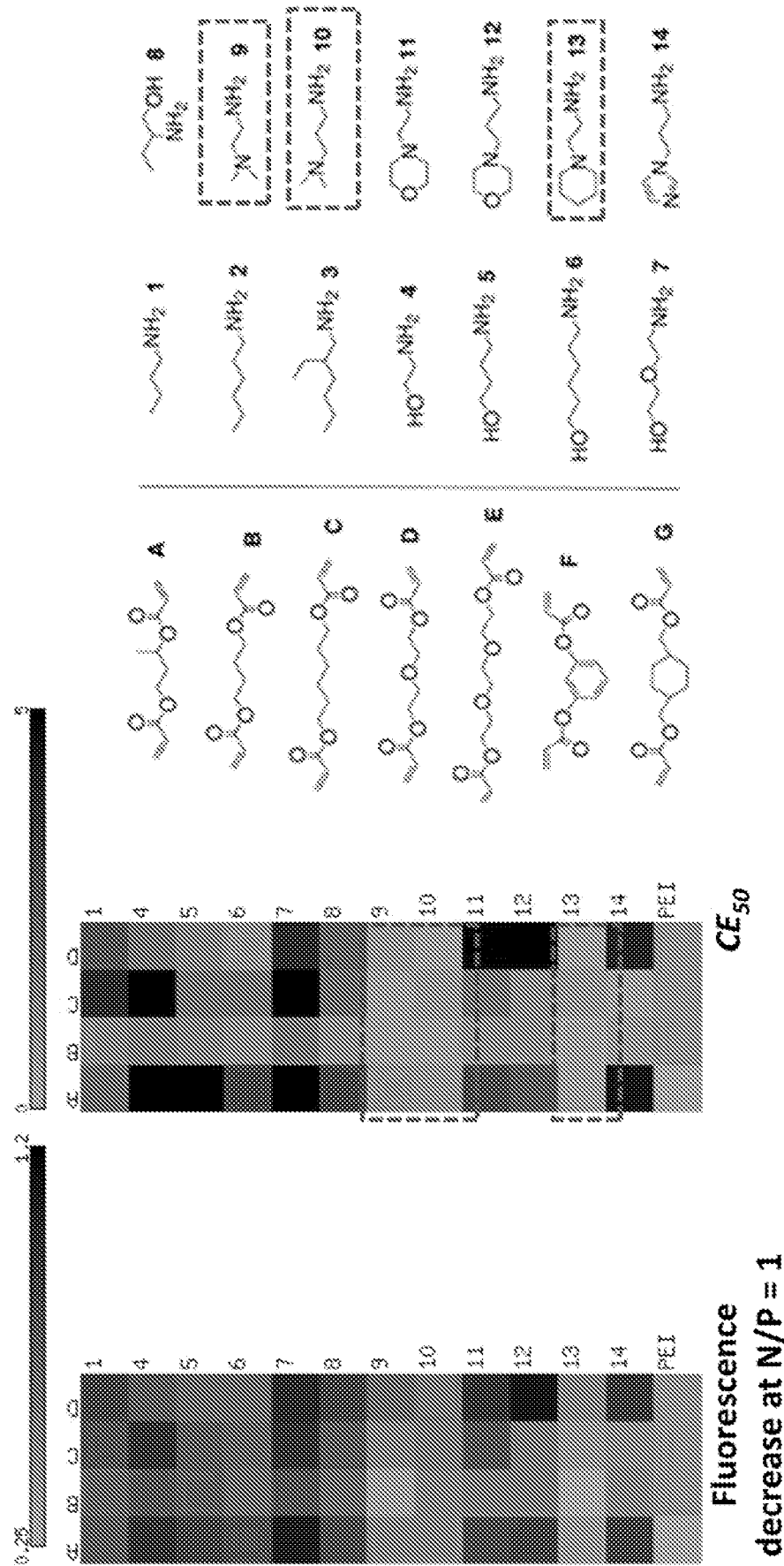


Figure 5A

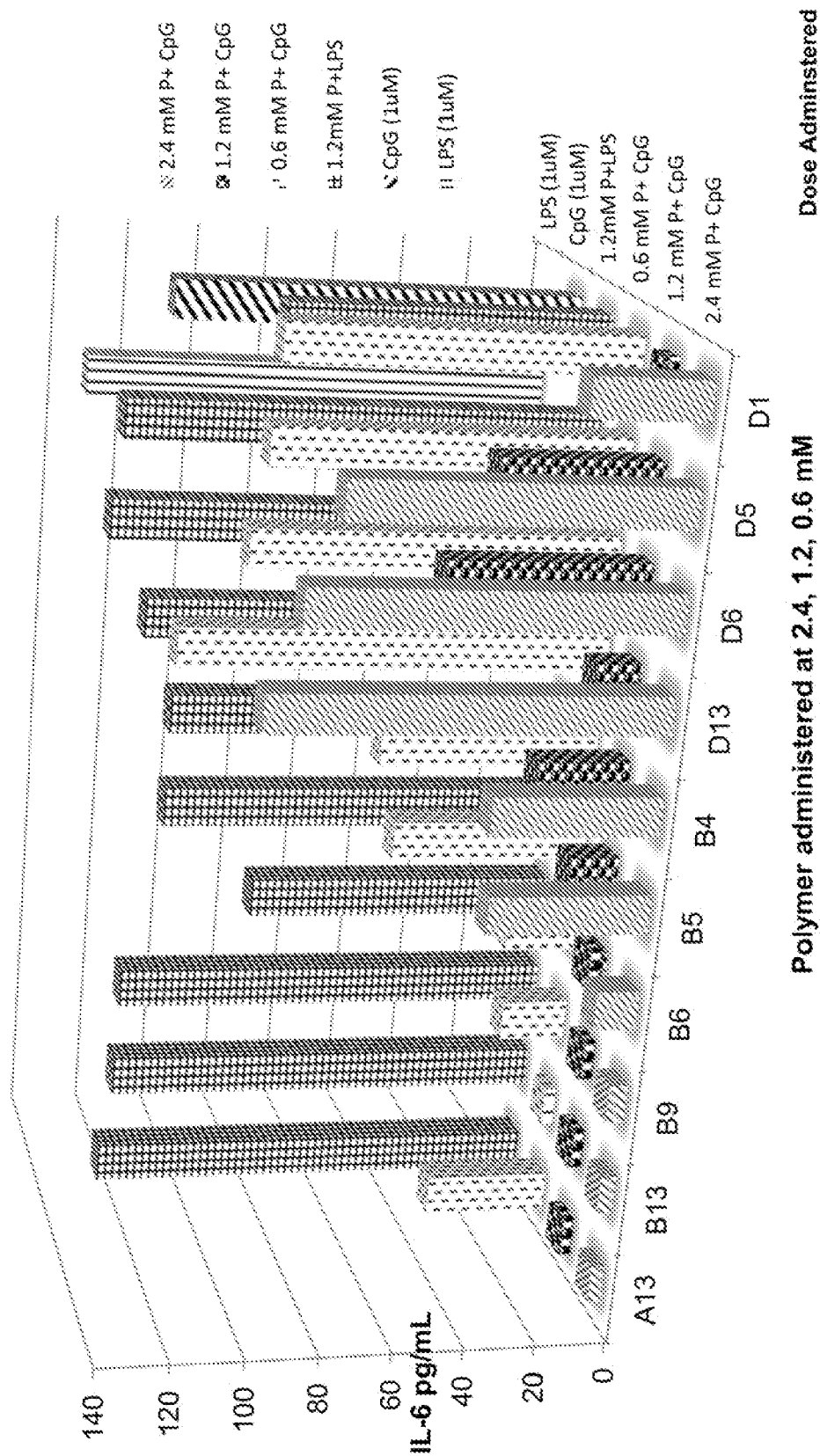


Figure 5B

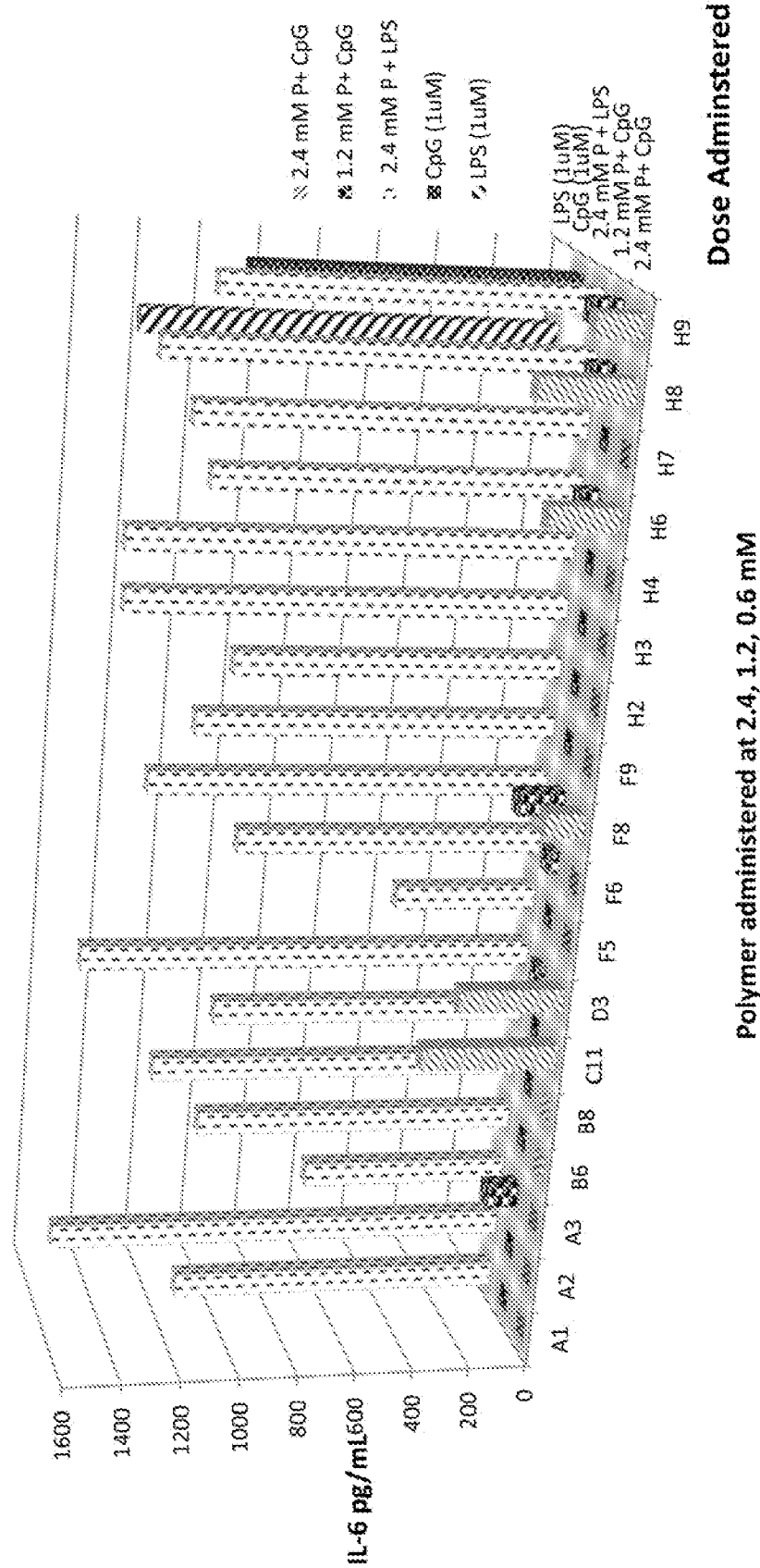


Figure 5C

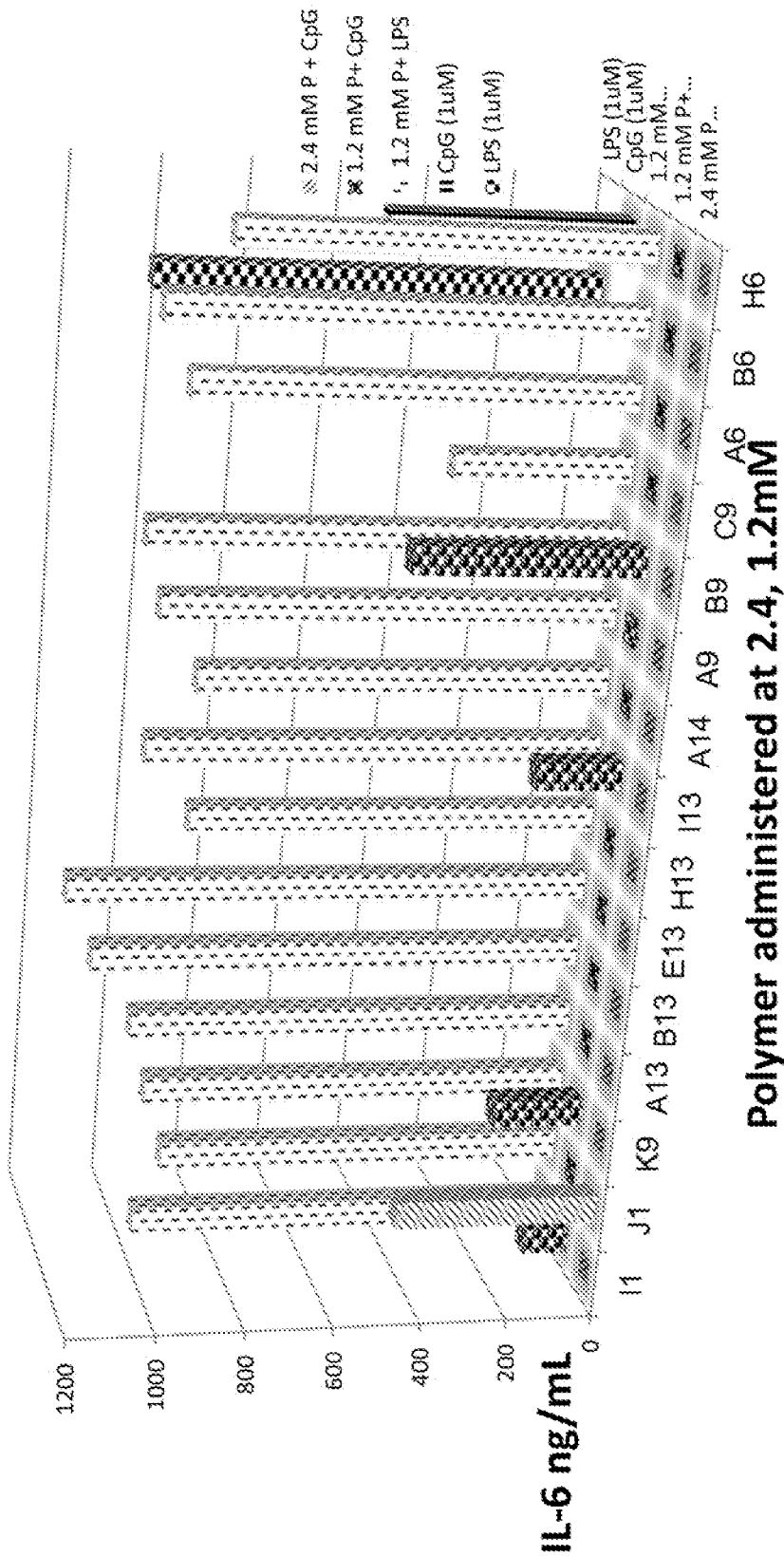


Figure 5D

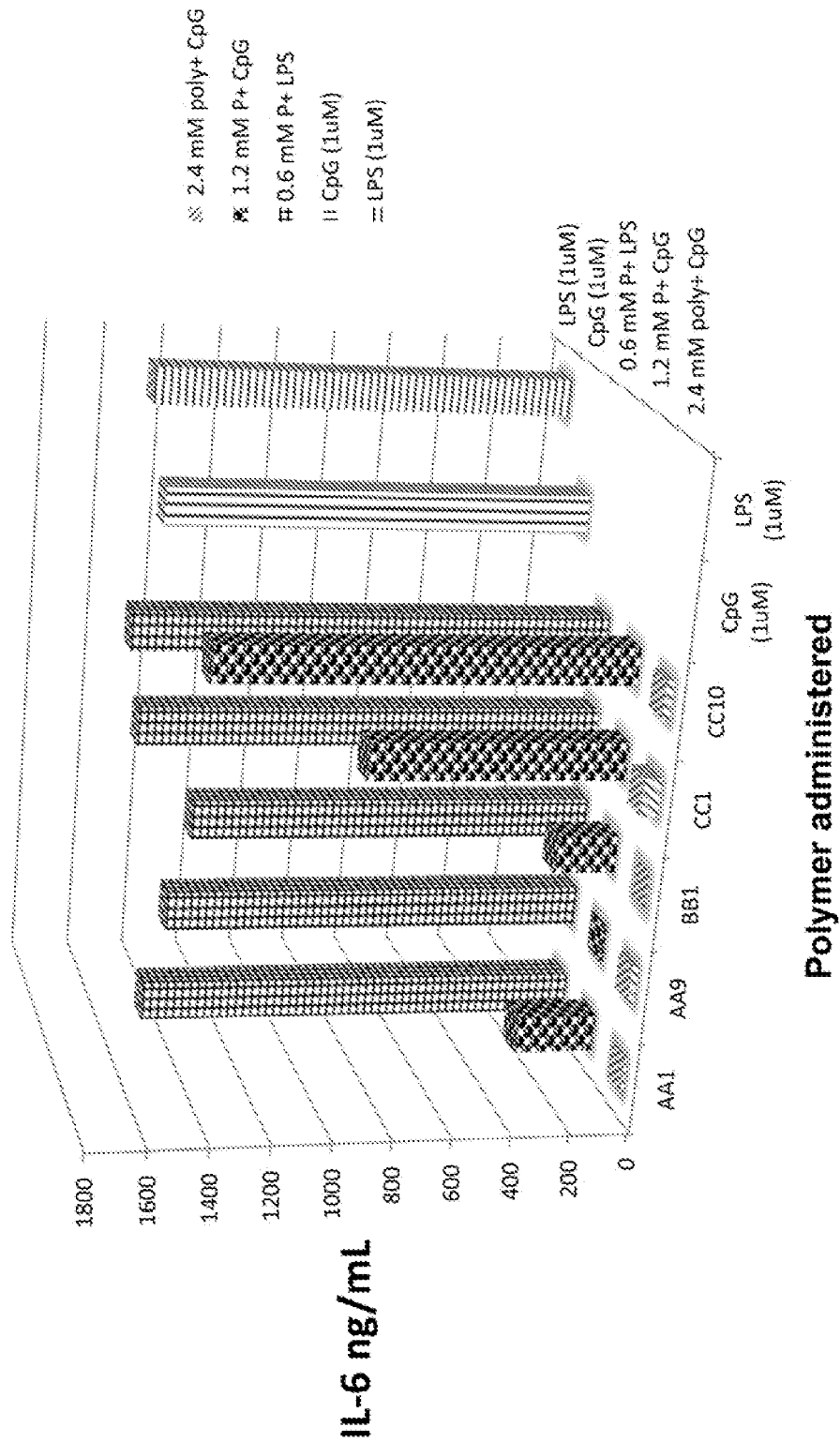


Figure 6A

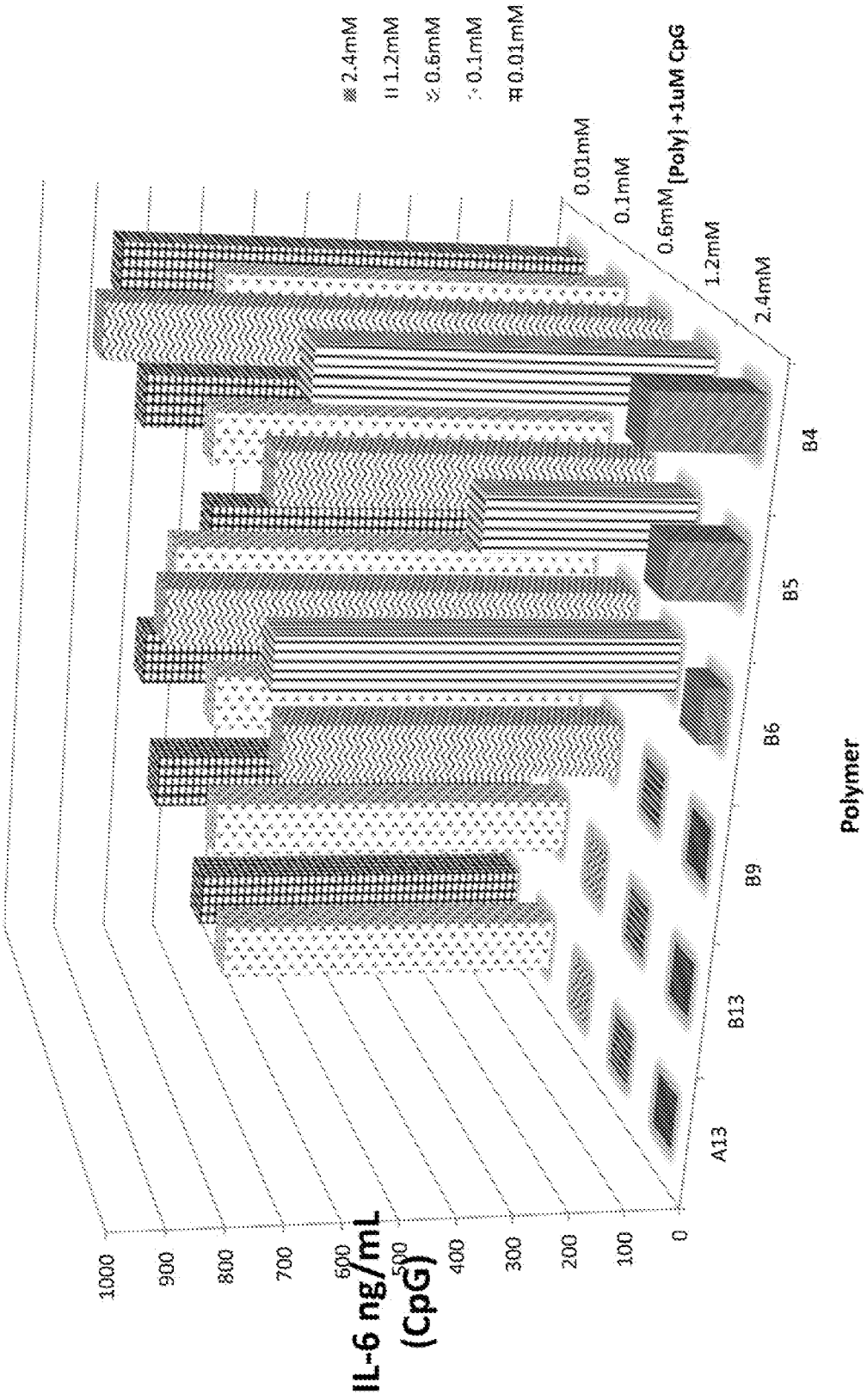


Figure 6B

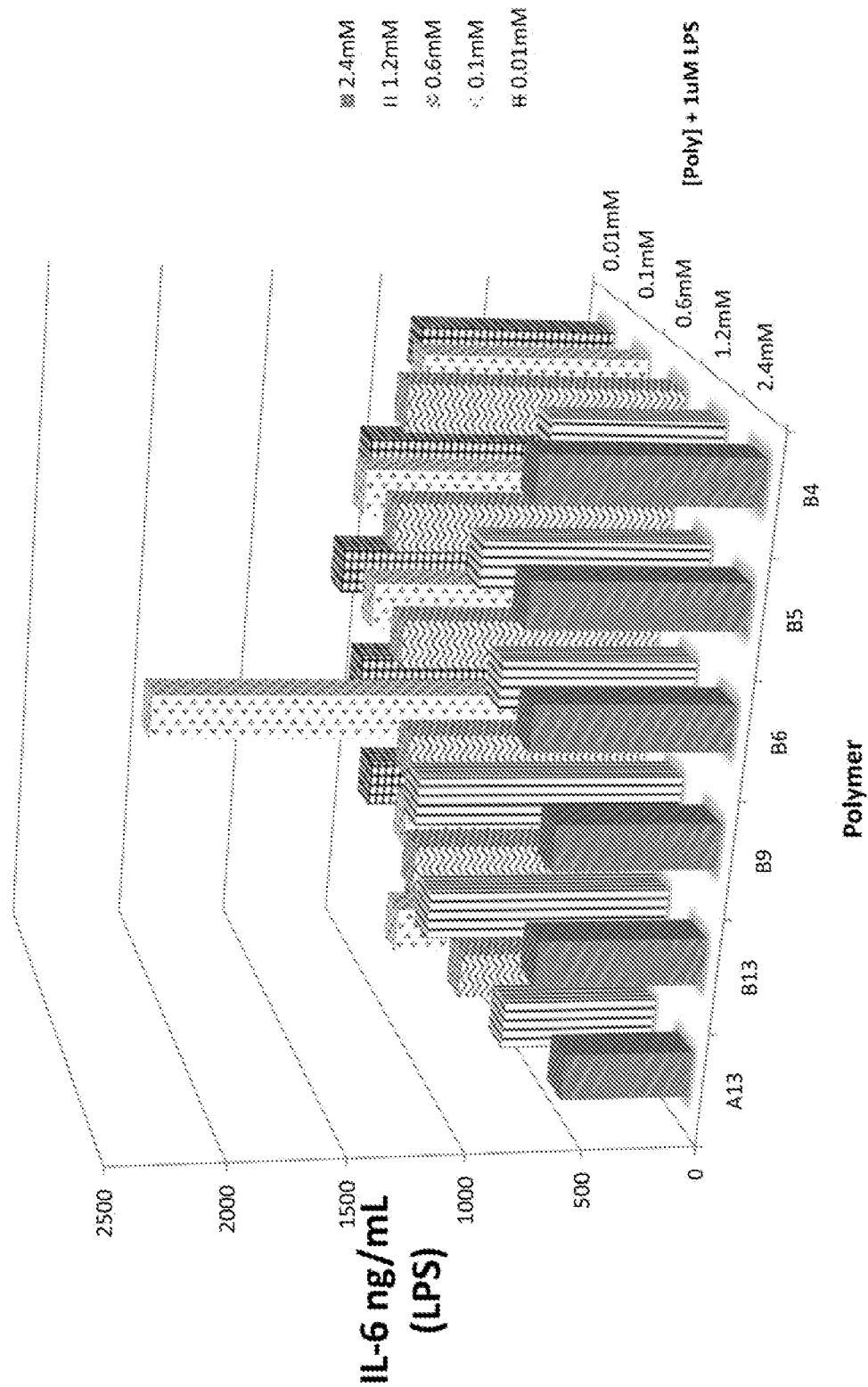


Figure 6C

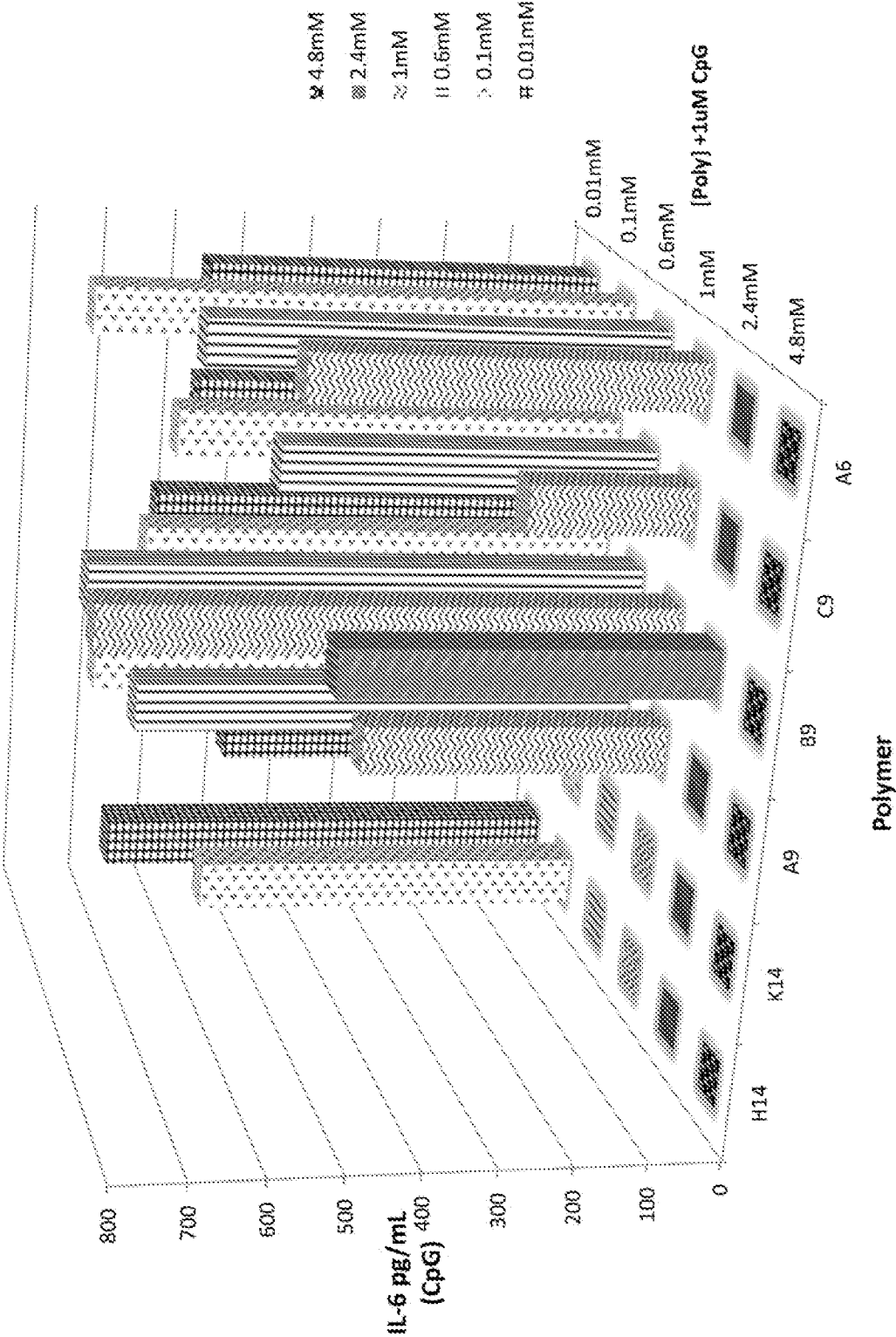


Figure 6D

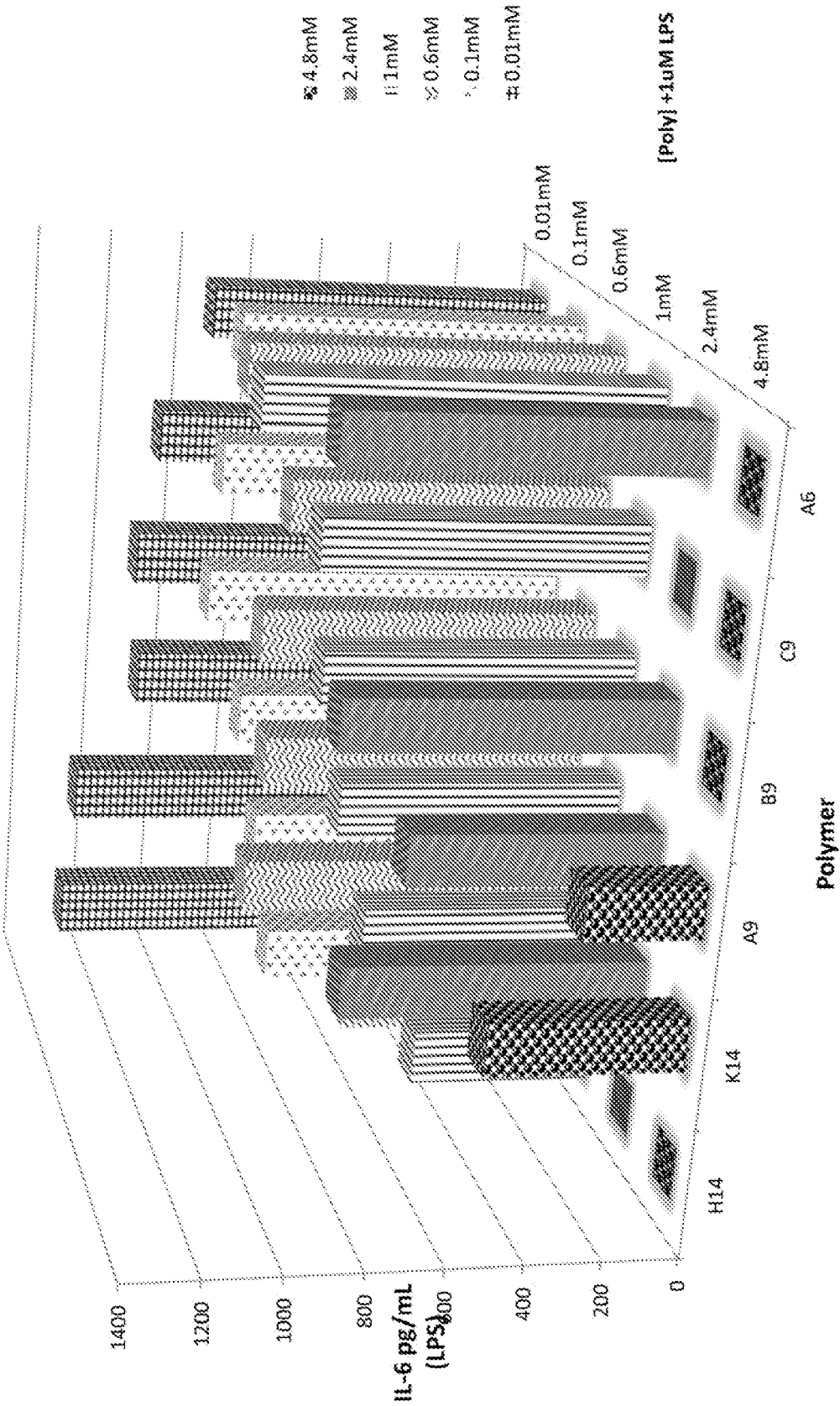


Figure 7A

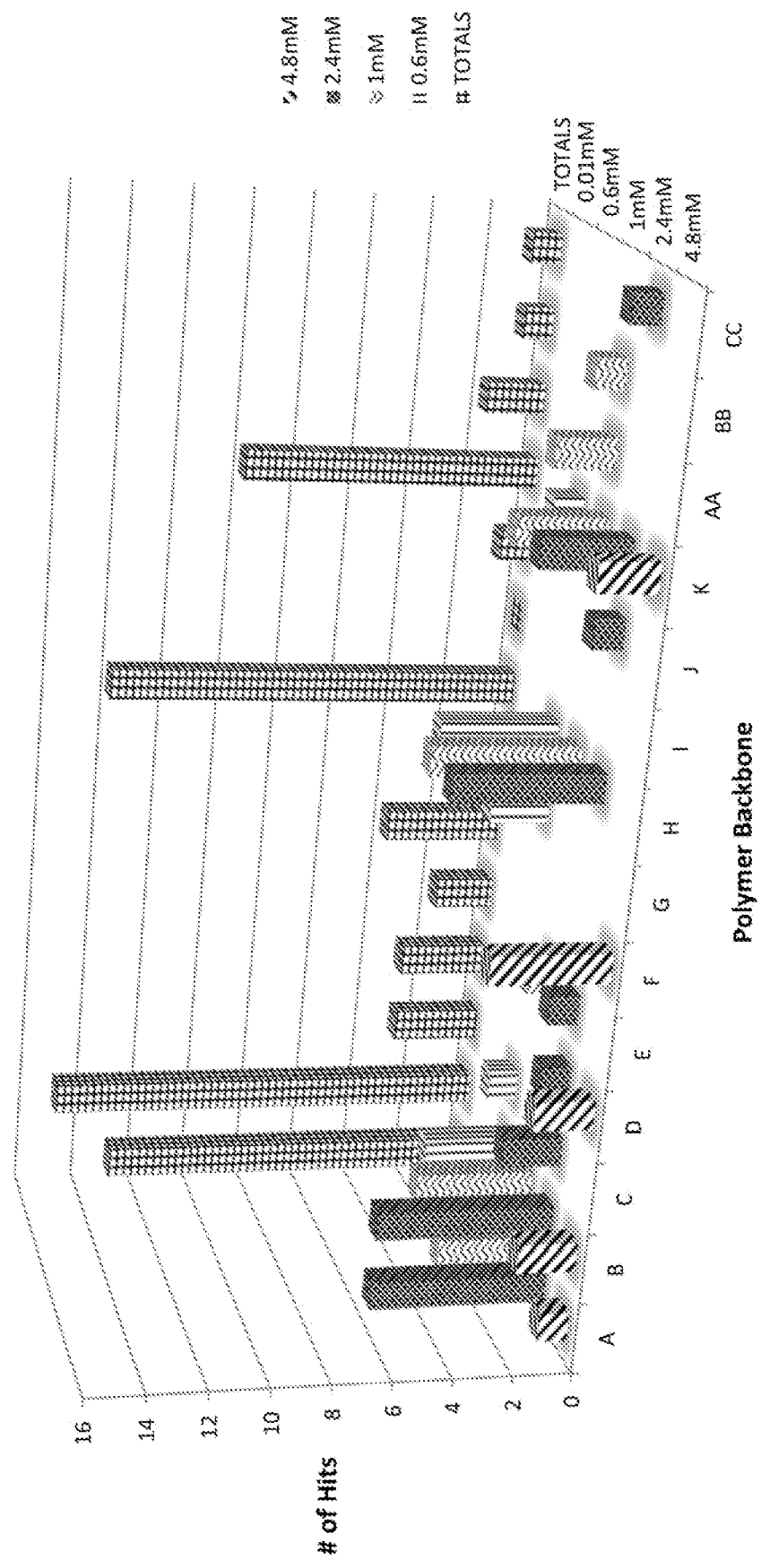


Figure 7B

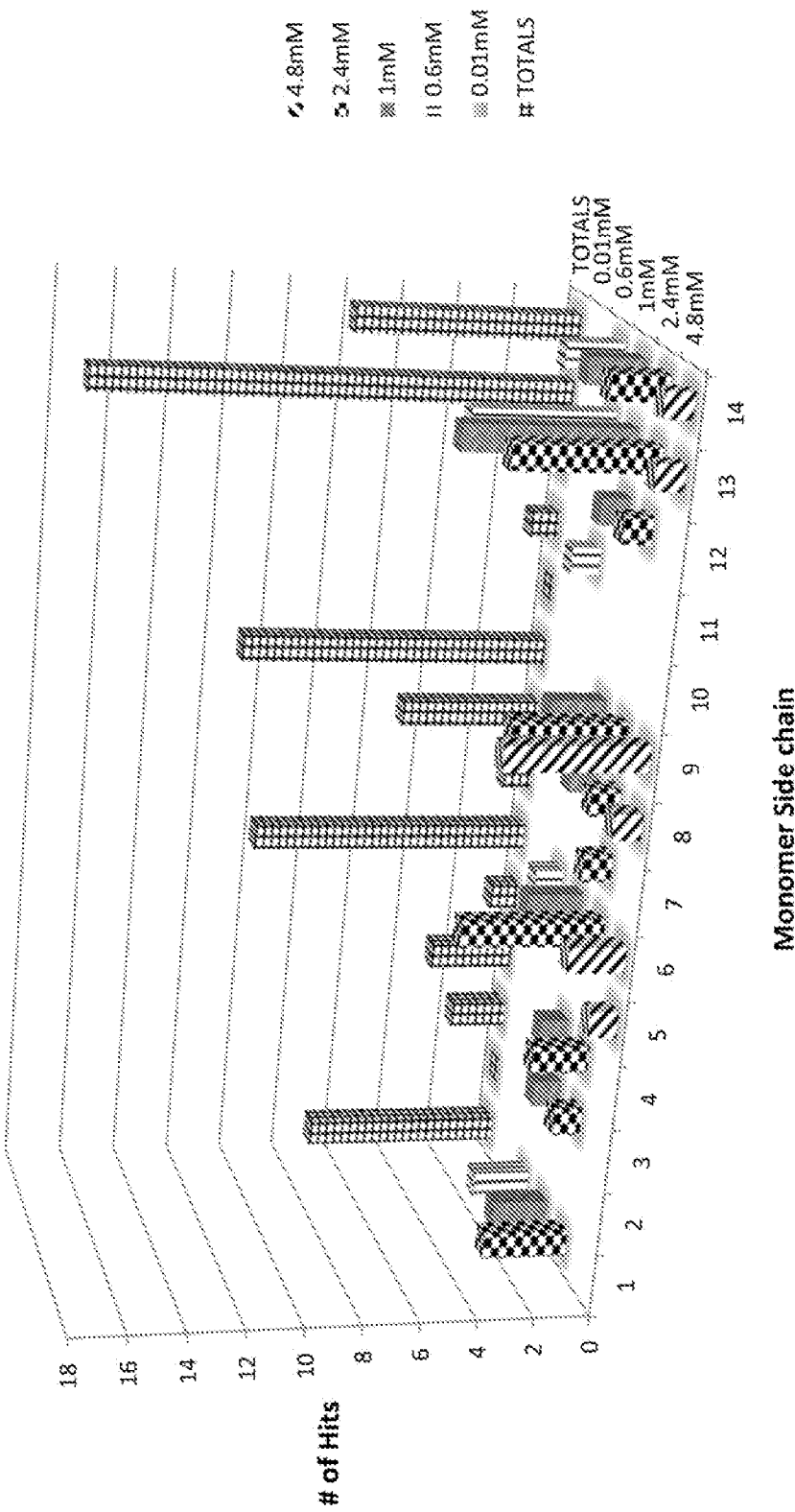


Figure 8

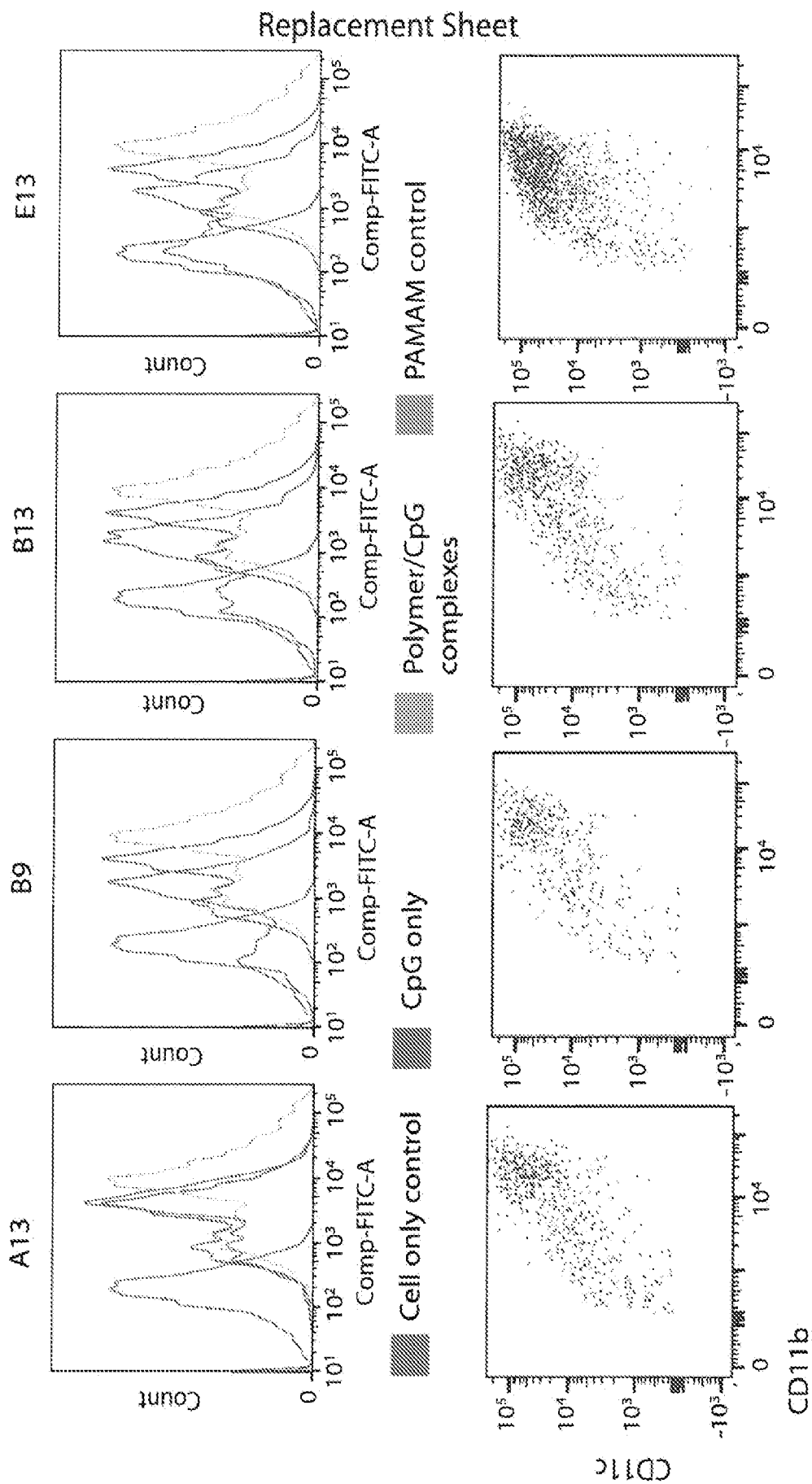
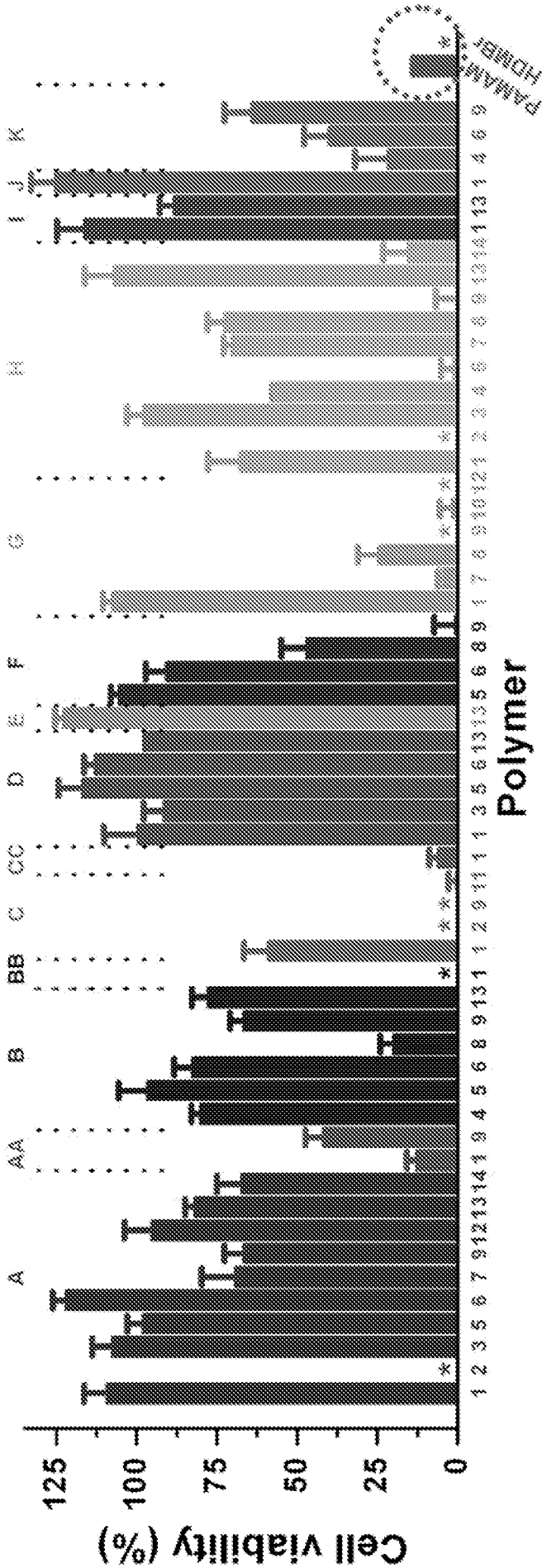


Figure 9



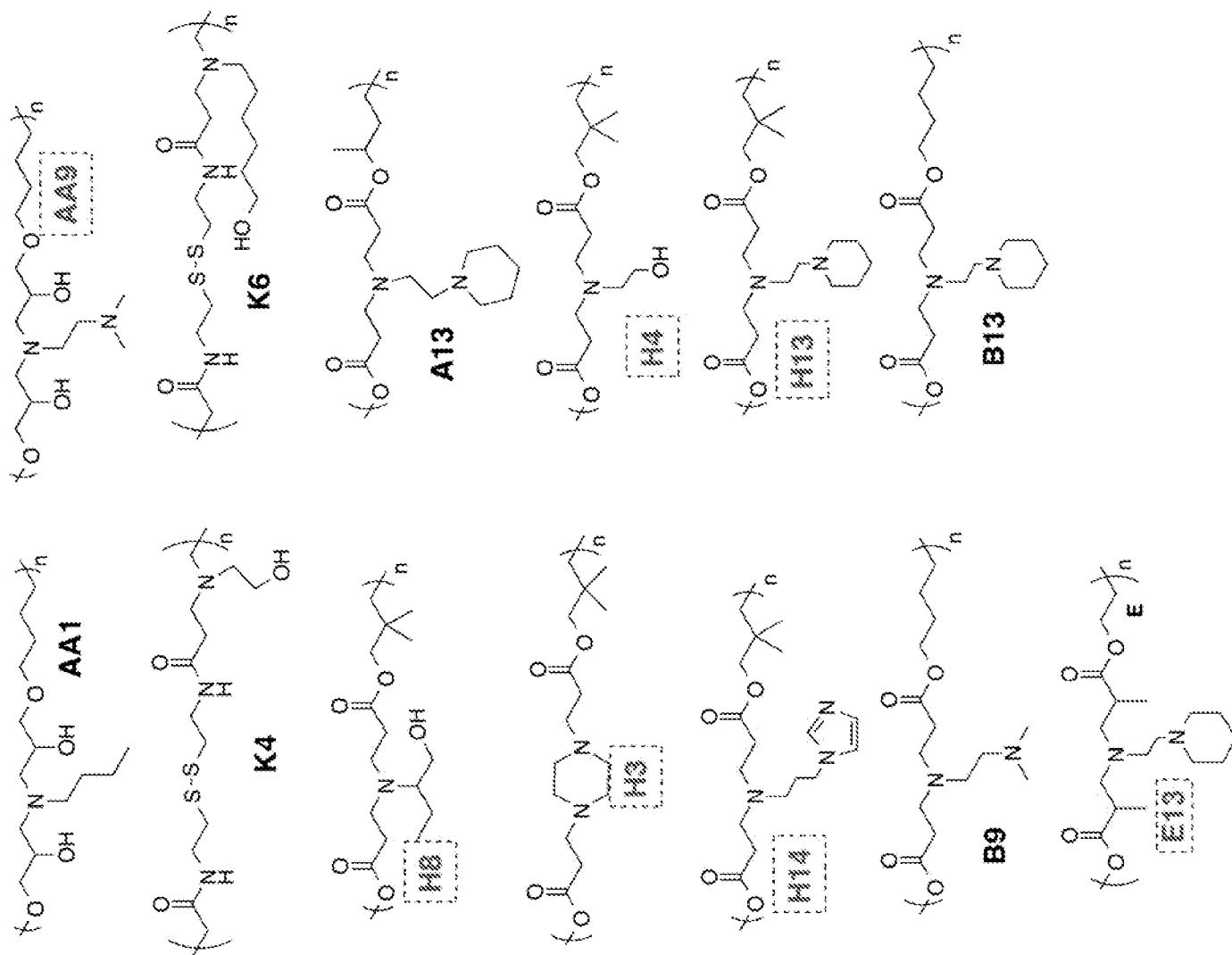


Figure 10

INTERNATIONAL SEARCH REPORT

PCT/US2014/033509 08-09-2014

International application No.

PCT/US2014/033509

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/16 (2014.01)

USPC - 424/172.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8) - A61K 31/16 31/407 31/70 31/722 31/785 38/02 38/14 38/16 38/17 39/395 A61P 3/04, 7/02, 9/00, 11/00, 17/06, 19/02, 25/00, 29/00, 31/00, 31/04, 35/00; C12Q1/68 (2014.01)

USPC - 424/172.1; 435/6.1; 514/1.1, 20.9, 55, 410, 616, 674

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

CPC - A61K 31/70, 38/16, 38/1709 (2014.06)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google, PubMed, SureChem

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/040552 A2 (MELLOR et al) 21 March 2013 (21.03.2013) entire document	1-3, 21, 29
Y		19, 20
Y	US 2012/0183564 A1 (SULLENGER) 19 July 2012 (19.07.2012) entire document	19
Y	US 2009/0298710 A1 (FAROKHZAD et al) 03 December 2009 (03.12.2009) entire document	20
A	US RE43,612 E (ANDERSON et al) 28 August 2012 (28.08.2012) entire document	1-3, 19-29
A	US 2012/0128782 A1 (GREEN et al) 24 May 2012 (24.05.2012) entire document	1-3, 19-29
P, X	HOLL et al. "Nucleic Acid Scavenging Polymers Inhibit Extracellular DNA-Mediated Innate Immune Activation without Inhibiting Anti-Viral Responses," Plos One, Vol. 8, Iss. 7, Pgs. 1-10. 23 July 2013 (23.07.2013) entire document	1-3, 19-29

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 August 2014

Date of mailing of the international search report

08 SEP 2014

Name and mailing address of the ISA/US

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Facsimile No. 571-273-3201

Authorized officer:

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300

PCT OSP: 571-272-7774

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.