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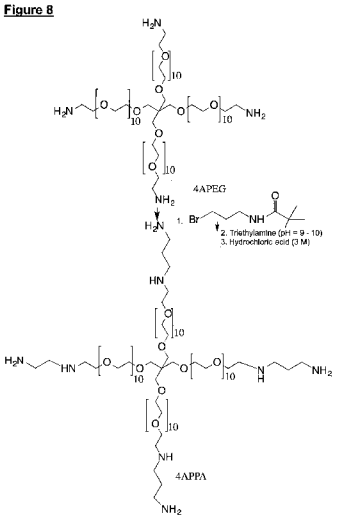
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(54) Title: GENE DELIVERY

Figure 8



(57) Abstract: The invention relates to a compound comprising poly(ethylene glycol) amine having a molecular weight of less than 5000 Daltons, wherein the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:44. The PEG amine compound is used as a novel delivery system for nucleic acids.

GENE DELIVERY

The present invention relates to nucleic acid or gene delivery, and to novel nucleic acid or gene delivery systems and vectors comprising polyethylene glycol amine. The invention extends to methods of preparing PEG amine/nucleic acid complexes, and to their uses for the delivery of nucleic acids, such as transgenes, in a variety of gene therapy applications. The invention further includes pharmaceutical compositions comprising such complexes.

Gene therapy was introduced as a concept in 1990. However, while the first gene therapeutic was introduced fairly rapidly thereafter in 2004, the pace of registration of gene therapeutics has been slow, despite significant capital investment and academic research interest. The prime reason for this has been the lack of effective gene delivery technologies.

Nucleic acids show a poor ability to access the cell nucleus (their site of activity) on intravenous injection due to their rapid degradation in the plasma and their inability to cross the endothelial, plasma and nuclear membranes. The three products that have received marketing authorisations: Gendicine, delivering the p-53 replacement gene, Rexin G delivering the cytotoxic cyclin G1 gene, and Glybera, delivering the lipoprotein lipase gene, all utilise viral vectors (adenovirus, lentivirus and adeno-associated viruses, respectively). This is despite the considerable effort that has gone into producing synthetic vectors. Furthermore, the only gene therapy product currently launched and available – Gendicine (Glybera is due to be launched in 2015) – is administered intratumourally. Furthermore, trials with Glybera involved dozens of intramuscular injections with each dose, as the muscle is the site of lipoprotein lipase production. These local injection choices suggest that there is thus an urgent need to provide systemically active gene therapies that are able to treat disseminated disease, such as metastatic neoplasms.

Synthetic vectors could enable such treatments, providing they cross the regulators' safety and efficacy bar. To date, all synthetic vectors have been amine rich materials that act by forming electrostatic complexes with nucleic acids and such complexes (dendriplexes in the case of dendrimers, and polyplexes in the case of polymers) enable the nucleic acids to be protected from degradation in the plasma and arrive at their cellular destination, where they are taken up by cells via endocytotic mechanisms and ultimately the nucleic acid cargo is delivered to the nucleus. The mechanism of uptake

is largely agreed to be by endocytosis, but the intracellular trafficking mechanisms are still hotly debated, with the ability to avoid endosomal acidity cited as being important by some and less so by others. Subsequent to endosomal escape, exogenous nucleic acids may enter the cell nucleus during cell division but entry post mitosis is extremely difficult and appears to take place via the nuclear pore complex.

A number of synthetic vectors have been developed based on polyamine molecules such as linear poly(ethylenimine) (PEI, molecular weight ~ 22 kDa) and the generation 2 poly(propylenimine) dendrimer (DAB 16, molecular weight = 1687 Da). However, these have not transitioned to clinical testing. One of the reasons for this is the relatively low biocompatibility of underivatised polyamines, as these have IC50 values (the concentration required to inhibit the growth of a cell population by 50%) in the low microgram per millilitre range, and PEI, for example, demonstrates severe toxicities *in vivo* on intravenous injection. The poor biocompatibility of these molecules has been attributed to the possible electrostatic interaction between the cationic complex and the anionic components of the plasma membrane. A further reason for the lack of clinical advancement of these polyamine synthetic gene therapy vectors, of which PEI has been the most studied, is that without derivatisation with targeting ligands, linear PEI targets the lung on intravenous injection. However, even though DAB 16 is able to avoid the lung on intravenous injection and ultimately produce tumour gene expression and indeed tumour regression, when loaded with a tumouricidal gene, the fact still remains that polyamines are relatively cytotoxic to cells. In actual fact various generations of the poly(propylenimine) dendrimers are anti-proliferative *in vivo* and cell cytotoxicity increases with molecular weight. While this anti-proliferative activity could be of benefit to cancer chemotherapy, gene therapy of other diseases will not be suited to the use of these dendrimers. Furthermore, even in cancer chemotherapy, a more biocompatible gene delivery system will ensure that off target effects stemming from the delivery system are not encountered and ultimately these factors will improve the therapeutic index of the medicine.

There is therefore a need for an improved gene delivery vector for use in gene therapy techniques. The inventors hypothesised that a low molecular weight polymer with reduced amine content would yield biocompatible gene therapy nanoparticles with nucleic acid. They therefore synthesised and characterised amine-terminated poly(ethylene glycol)-based polymers, and subjected them to physicochemical and biological testing. They found that the new PEG derivative is 1000X less toxic than

poly(ethylenimine) in a human carcinoma cell line, has comparable cell transfection capability to PEI in this cell line and produces tumouricidal nanoparticles when complexed with TNF-alpha.

Hence, in a first aspect, there is provided a compound comprising poly(ethylene glycol) amine having a molecular weight of less than 5000 Daltons, wherein the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:44.

The inventors have developed several embodiments of novel PEG amine compounds, which can be combined with nucleic acids (e.g. siRNA, mRNA or DNA) to form novel biocompatible complexes. The PEG amine compounds complex with nucleic acids via electrostatic interactions between the cationic charge of the compound and the anionic charge of the nucleic acid phosphate groups, thereby forming positively charged nanoparticles. Advantageously, the polymer protects the nucleic acids from degradation and achieves *in vitro* transfection/silencing that is comparable to that achieved by Lipofectamine and the generation 3 poly(propylenimine) dendrimer (DAB 16), despite being 2-3 orders of magnitude less toxic than these agents. Advantageously, as described in the Examples, these complexes may be used to produce efficient nucleic acid or gene delivery systems, which are non-toxic to host cells.

Thus, in a second aspect, there is provided use of the compound according to the first aspect, as a nucleic acid delivery system.

It will be appreciated that an ethylene glycol unit is: $-CH_2CH_2O-$.

Preferably, the compound has a molecular weight of less than 4000 Daltons, more preferably less than 3000 Daltons, and most preferably less than 2500 Daltons.

Preferably, the compound has a molecular weight of at least 500 Daltons, more preferably at least 1000 Daltons, and most preferably at least 1500 Daltons.

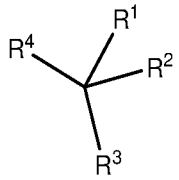
Preferably, the compound has a molecular weight between 500 and 4000 Daltons, more preferably between 1000 and 3000 Daltons, and most preferably between 1500 and 2500 Daltons.

Preferably, the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:40. More preferably, the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:35, between 30:3 and 30:30, between 30:3 and 30:25, between 30:3 and 30:20, or between 30:3 and 30:15. Most preferably, the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:10.

Preferably, the ratio of ethylene glycol units to nitrogen atoms is between 30:10 and 30:44. More preferably, the ratio of ethylene glycol units to nitrogen atoms is between 30:15 and 30:44, between 30:20 and 30:44, between 30:25 and 30:44, between 30:30 and 30:44, or between 30:35 and 30:44. Most preferably, the ratio of ethylene glycol units to nitrogen atoms is between 30:40 and 30:44.

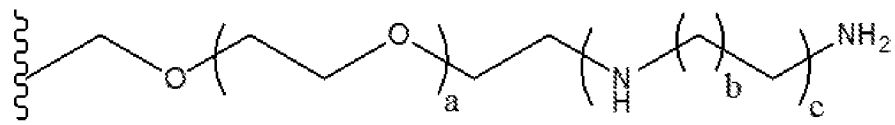
However, in a most preferred embodiment, the ratio of ethylene glycol units to nitrogen atoms is about 30:6.

The compound of the first aspect may have Formula (I):



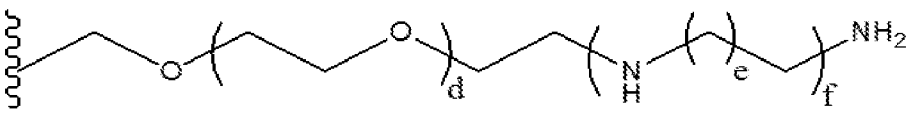
Formula (I)

wherein R^1 is hydrogen or:



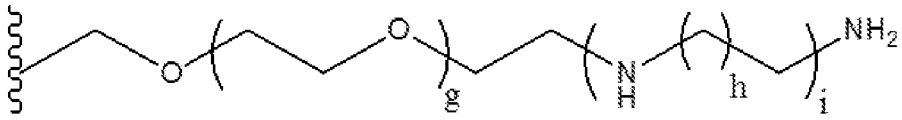
wherein a is an integer between 1 and 42,
b is 1 or 2, and
c is an integer between 0 and 10;

R^2 is hydrogen or:



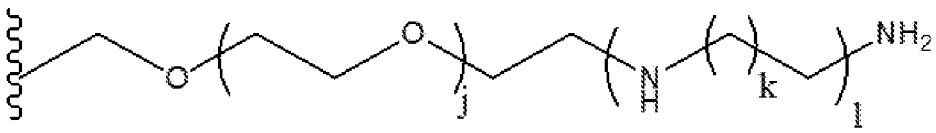
wherein d is an integer between 1 and 42,
e is 1 or 2, and
f is an integer between 0 and 10;

R^3 is hydrogen or:



wherein g is an integer between 1 and 42,
h is 1 or 2, and
i is an integer between 0 and 10; and

R^4 is hydrogen or:



wherein j is an integer between 1 and 42,
k is 1 or 2, and
l is an integer between 0 and 10;
wherein the sum of a, d, g and j is between 30 and 50 and the sum of c, f, i and l is between 1 and 40.

It will be understood that in order to comply with the definition of Formula (I) at least one of R^1, R^2, R^3 and R^4 must not be hydrogen, *i.e.* at least one of R^1, R^2, R^3 and R^4 must comprise a straight chain alkyl, as defined above.

Accordingly, three of R^1, R^2, R^3 and R^4 may be hydrogen and one of R^1, R^2, R^3 and R^4 may be a straight chain alkyl. However, in a preferred embodiment, two of R^1, R^2, R^3 and R^4 are hydrogen and two of R^1, R^2, R^3 and R^4 are a straight chain alkyl. In a more preferred embodiment, one of R^1, R^2, R^3 and R^4 is hydrogen and three of R^1, R^2, R^3 and R^4 are a straight chain alkyl. In a most preferred embodiment, none of R^1, R^2, R^3 and R^4 are hydrogen and each of R^1, R^2, R^3 and R^4 are a straight chain alkyl.

Preferably, a is an integer between 2 and 30, more preferably between 3 and 25, even more preferably between 4 and 20, and most preferably between 5 and 15. Preferably, a is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. In a most preferred embodiment, however, a is 10.

Preferably, d is an integer between 2 and 30, more preferably between 3 and 25, even more preferably between 4 and 20, and most preferably between 5 and 15. Preferably, d is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. In a most preferred embodiment d is 10.

Preferably, g is an integer between 2 and 30, more preferably between 3 and 25, even more preferably between 4 and 20, and most preferably between 5 and 15. Preferably, g is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. In a most preferred embodiment g is 10.

Preferably, j is an integer between 2 and 30, more preferably between 3 and 25, even more preferably between 4 and 20, and most preferably between 5 and 15. Preferably, j is 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. In a most preferred embodiment j is 10.

Preferably, c is an integer between 0 and 5. Preferably, c is 0, 1, 2, 3, 4 or 5. More preferably, c is between 1 and 4. In a most preferred embodiment c is 1.

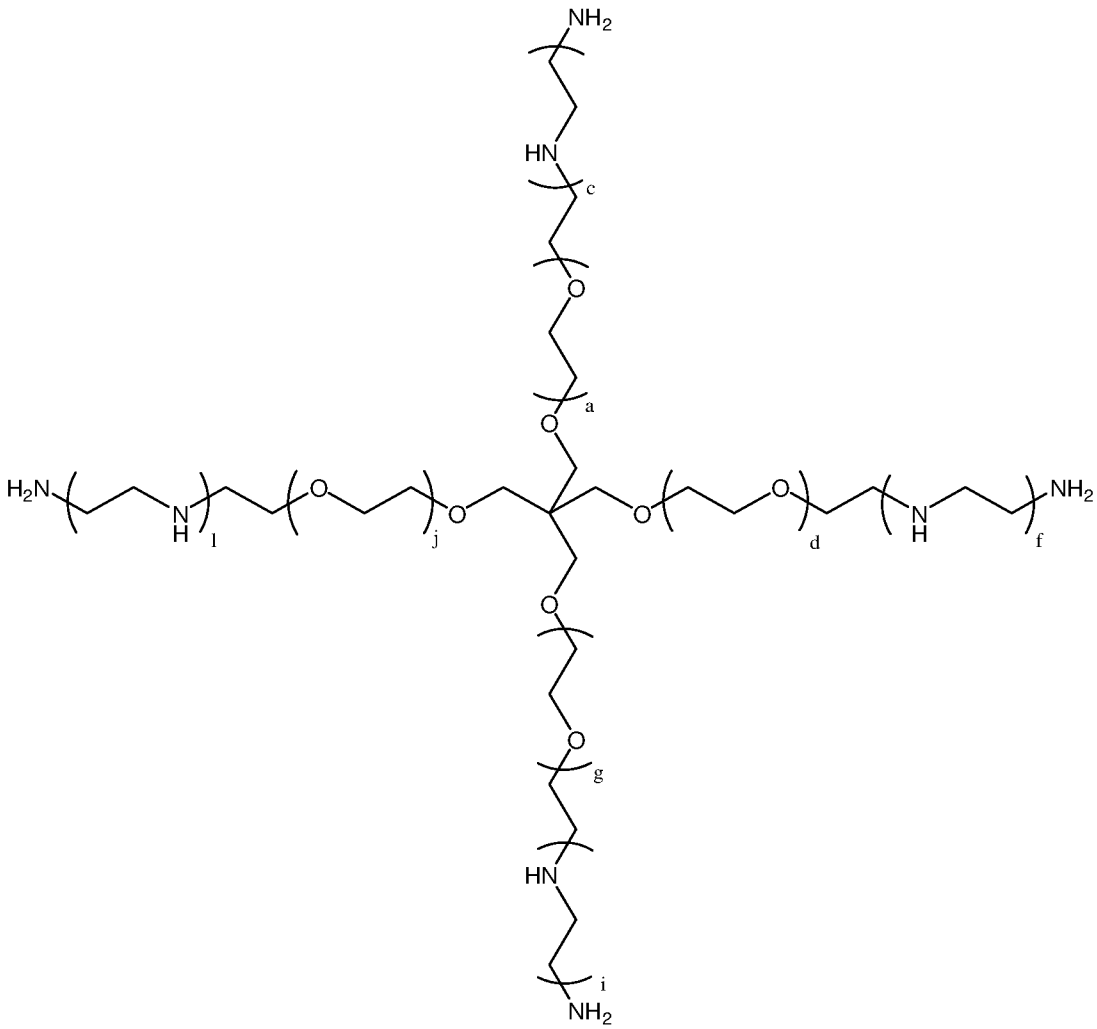
Preferably, f is an integer between 0 and 5. Preferably, f is 0, 1, 2, 3, 4 or 5. More preferably, f is between 1 and 4. In a most preferred embodiment f is 1.

Preferably, i is an integer between 0 and 5. Preferably, i is 0, 1, 2, 3, 4 or 5. More preferably, i is between 1 and 4. In a most preferred embodiment i is 1.

Preferably, l is an integer between 0 and 5. Preferably, l is 0, 1, 2, 3, 4 or 5. More preferably, l is between 1 and 4. In a most preferred embodiment l is 1.

Preferably, the sum of c, f, i and l is between 1 and 30, more preferably between 2 and 20, and most preferably between 4 and 10.

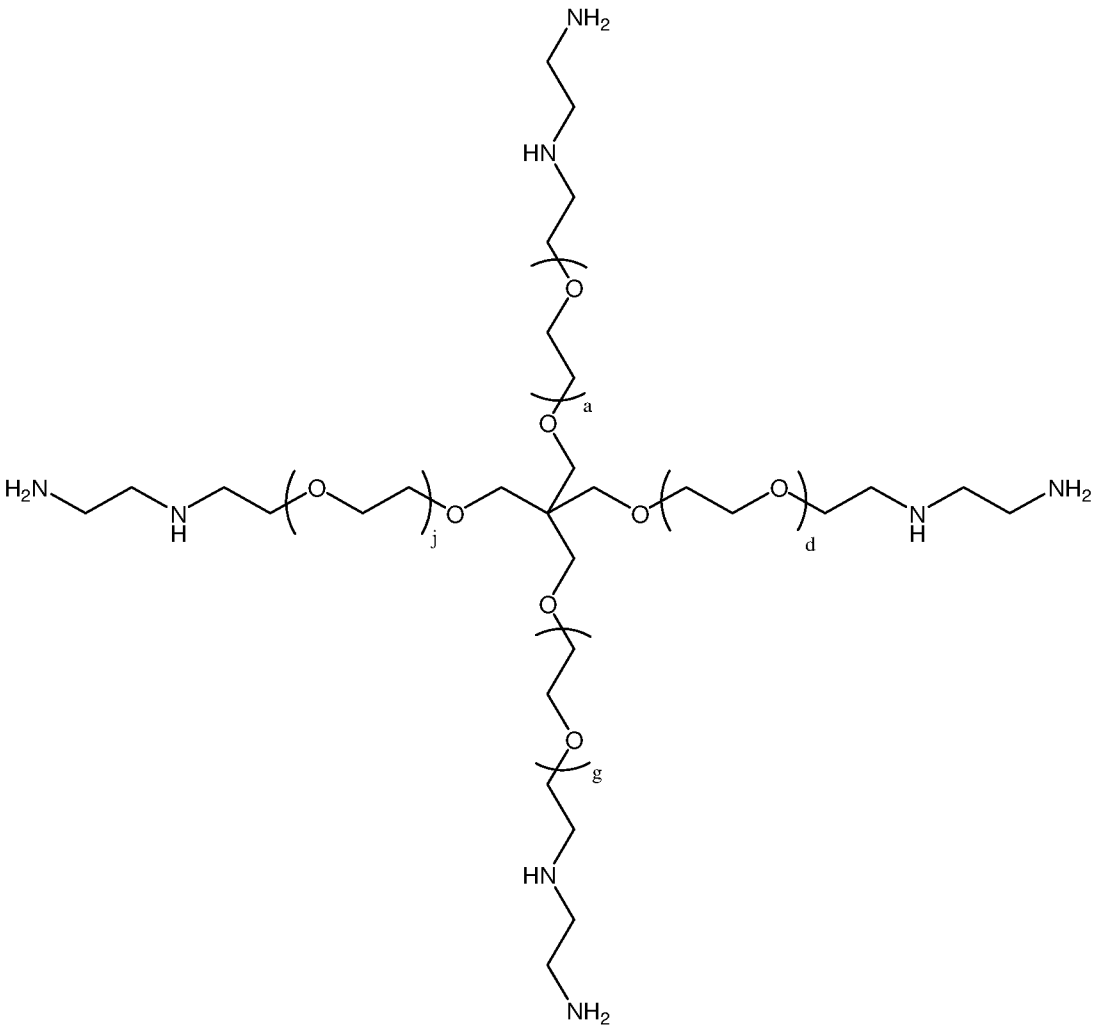
In one preferred embodiment, b is 1, e is 1, h is 1 and k is 1. Accordingly, the compound preferably has Formula (Ia):



Formula (Ia).

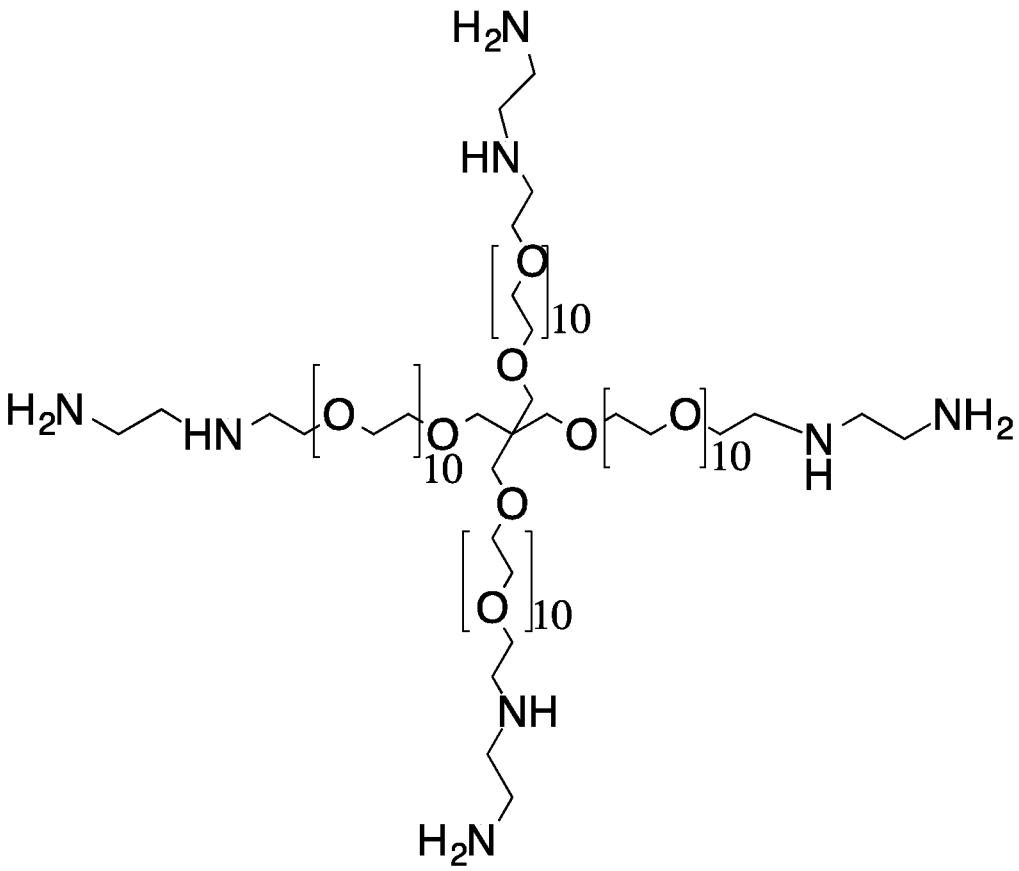
Preferably, the compound of Formula (Ia) has a molecular weight between 500 and 4000 Daltons, more preferably between 1000 and 3000 Daltons, and most preferably between 1500 and 2500 Daltons.

Preferably, c is 1, f is 1, i is 1 and l is 1. Accordingly, the compound preferably has Formula (Ib):



Formula (Ib).

Most preferably, the compound has Formula (Ic):

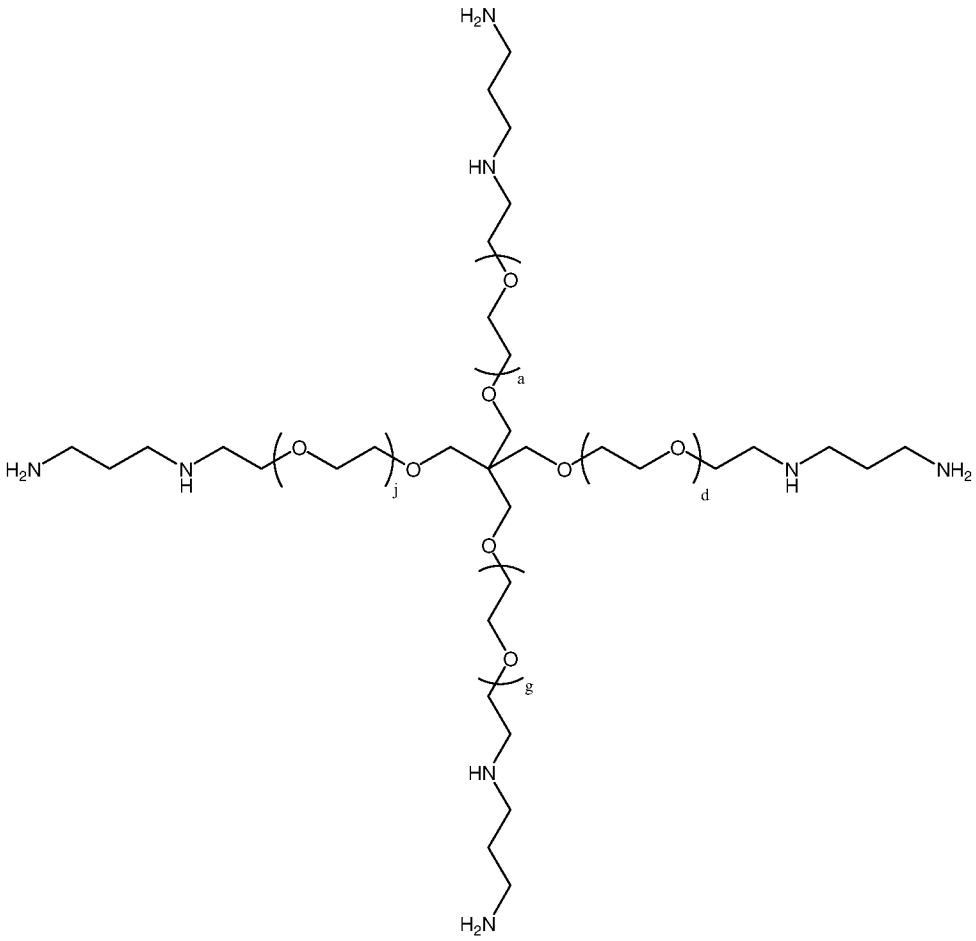


Formula (Ic).

Formula (Ic) is referred to herein as tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(2-ethylamine)-]-pentaerythritol, *i.e.* 4-arm-PEG-ethylamine or 4APEA.

As described in Example 1, the 2 kDa star shaped poly(ethylene glycol) (PEG) based amine contains at least 8 amine groups per molecule, shows good biocompatibility with IC50 values in excess of 4 mg mL^{-1} (Table 2) and is able to condense DNA and protect it from serum degradation (Figure 4d). The compound has been shown to efficiently transfer DNA into cells (Figure 5). The PEG amine diverts DNA to the liver and lung on intravenous administration (Figure 6). Tumouricidal gene PEG amine complexes are tumouricidal in a mouse pancreatic cancer xenograft models (Figure 7).

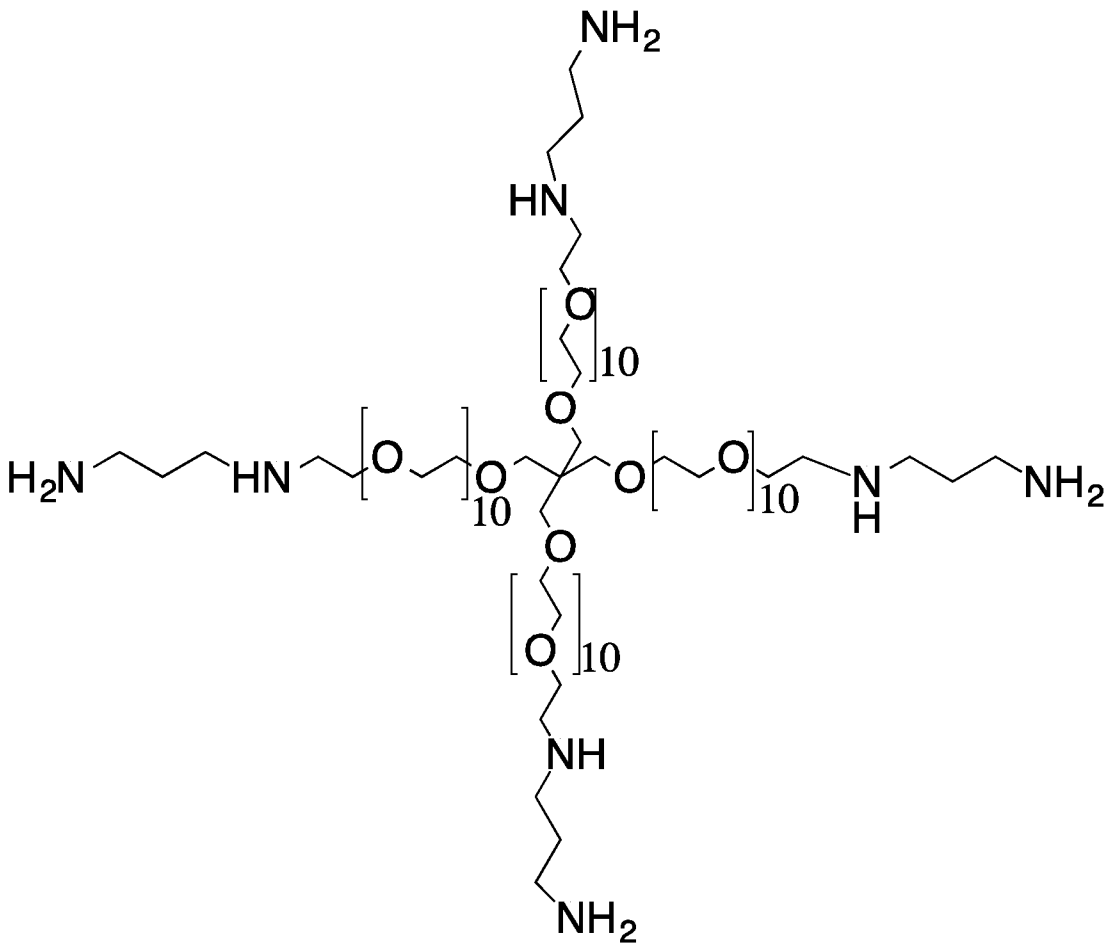
In an alternative preferred embodiment, b is 2, e is 2, h is 2 and k is 2. Preferably, c is 1, f is 1, i is 1 and l is 1. Accordingly, the compound may have Formula (Id):



Formula (Id).

Preferably, the compound of Formula (Id) has a molecular weight between 500 and 4000 Daltons, more preferably between 1000 and 3000 Daltons, and most preferably between 1500 and 2500 Daltons.

Preferably, the compound has Formula (Ie):



Formula (Ie).

Formula (Ie) is referred to herein as tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(3-propylamine)-]-pentaerythritol, i.e. 4PPA.

As described in Example 2, 4PPA was synthesised, characterised and complexed with a reporter gene/siRNA (beta-galactosidase plasmid DNA/siRNA-ITCH). The *in vitro* cell cytotoxicity of the PEG-amine-nucleic acid complexes were determined in A431 and MiaPaCa2 cell lines (Tables 4 and 5). The polymer complexes siRNA, encapsulating the siRNA within the complexed nanoparticles. Surprisingly, the polymer is over 400-fold less toxic than the third generation poly(propylenimine) dendrimer (DAB 16) and Lipofectamine. The polymer-gene/siRNA complex efficiently transfects cells in culture, as shown in Table 6 and Figure 11. The polymer – siRNA complex effectively transfers siRNA to the brain on intranasal administration (Figures 12 and 13). As described in Example 3, 4PPA was synthesised, characterised and complexed with a reporter mRNA (beta-galactosidase mRNA).

The inventors have developed a novel method for preparing a nucleic acid delivery complex or vector.

In a third aspect, there is provided a method of producing a nucleic acid delivery complex, the method comprising contacting the compound of the first aspect with a nucleic acid to thereby form a stable nucleic acid delivery complex.

In a fourth aspect, there is provided a nucleic acid delivery complex comprising the compound of the first aspect and a nucleic acid.

In one embodiment of the invention, the nucleic acid may be DNA, which may be genomic DNA or cDNA. For example, the nucleic acid may encode beta-galactosidase or TNF-alpha. As shown in Figure 7, on intravenous injection of the 4APEA – TNF-alpha gene complex to a pancreatic cancer xenograft mouse model, animals dosed with the 4APEA – TNF-alpha gene complex produced a tumouricidal response, with tumour volume significantly decreased relative to untreated controls.

In another embodiment, the nucleic acid may be RNA, such as antisense RNA, mRNA, siRNA or shRNA. For example, the nucleic acid may comprise siRNA against Itchy E3 Ubiquitin protein ligase (ITCH), i.e. 5'-CCACAACACACGAAUUACA-3' (SEQ ID No: 1).

As shown in Figure 13, on intranasal administration of 4PPA – siRNA-ITCH, down regulation of the ITCH gene was seen in the brains of said animals.

The PEG amine - nucleic acid complex may be prepared by mixing the nucleic acid and PEG amine in a suitable buffer (e.g. phosphate buffer solution, 2mM, pH 6) and allowing the mixture to incubate at room temperature for at least 1 hour, to ensure that the dynamic stability of the complex is reached. However, the skilled person would appreciate that the length of time that the PEG amine is incubated with the nucleic acid depends upon the temperature at which the incubation occurs, the type of PEG amine and the type of nucleic acid. For instance, at higher temperatures, such as 25-30°C, the incubation step can be at least 10 minutes, while at lower temperatures, such as 20-25°C, the incubation step can be at least 15 minutes. Any of the temperatures described herein may be combined with any of the incubation times described herein.

The ratio of PEG amine to nucleic acid mixed may be measured as a ratio of nitrogen (PEG amine) to phosphate (DNA) ratios (N, P ratios). The N:P ratio may be between 1:1 and 500:1, between 2:1 and 400:1, between 3:1 and 300:1, between 4:1 and 250:1 or between 5:1 and 200:1. Preferably, the N:P ratio is between 6:1 and 180:1, or between 7:1 and 160:1, between 8:1 and 140:1, or between 9:1 and 120:1 or between 10:1 and 100:1. More preferably, the N:P ratio is between 15:1 and 80:1, or between 20:1 and 60:1, between 25:1 and 55:1, or between 30:1 and 50:1 or between 35:1 and 45:1.

Alternatively, the ratio of PEG amine to nucleic acid mixed in the buffer may be measured as a weight ratio. The weight ratio of the PEG amine to the nucleic acid mixed in the buffer may be between 1:1 and 100:1, between 2:1 and 50:1, between 3:1 and 45:1, between 4:1 and 40:1, or between 5:1 and 35:1. Preferably, the weight ratio of the PEG amine to the nucleic acid is between 6:1 and 30:1, between 7:1 and 26:1, between 8:1 and 25:1, between 9:1 and 24:1, or between 10:1 and 23:1. More preferably, the weight ratio of the PEG amine to the nucleic acid is between 11:1 and 22:1, between 12:1 and 21:1, between 13:1 and 20:1, or between 14:1 and 19:1, or between 15:1 and 18:1. Most preferably, the weight ratio of the PEG amine to the nucleic acid is about 16:1.

As explained in the Examples, the PEG amine forms a complex with the nucleic acid. Preferably, the zeta-potential of the PEG amine -nucleic acid complex is at least + 5 mV at neutral pH and does not exceed + 90 mV at neutral pH.

It will be understood that the pH may be considered to be neutral if it is between 6.5 and 7.5.

The PEG amine - nucleic acid complex may be in the form of a nanoparticle. Preferably, the average diameter of nanoparticle is more than about 100nm, more preferably more than about 150, and most preferably more than about 200nm. Preferably, the average diameter of nanoparticle is less than about 900nm, more preferably less than about 700, even more preferably less than about 500 nm, and most preferably less than about 300nm. Most preferably, the average diameter of nanoparticle is about 200nm-300nm.

The inventors have demonstrated that the PEG amine -nucleic acid complexes of the invention can be efficiently targeted to, and transduce tumour cells, which are subsequently killed. However, it will be appreciated that the type of cell, which is targeted by the complex can vary depending on the nucleic acid. In the Examples,

tumour cells are used illustratively to show that the complex of the invention exhibit a significantly improved transduction.

Thus, in a fifth aspect, there is provided the compound of the first aspect or the complex according to the fourth aspect, for use in therapy or diagnosis.

The invention may be used for the treatment of a wide variety of diseases due to the target-specific nature and the improved transduction efficiency of the complex of the invention. The invention may be used prophylactically to prevent disease, or after the development of a disease, to ameliorate/treat it.

Hence, in a sixth aspect, there is provided the compound according to the first aspect or the complex according to the fourth aspect, for use in a gene therapy technique.

In a seventh aspect, there is provided a method of treating, preventing or ameliorating a disease in a subject using a gene therapy technique, the method comprising administering, to a subject in need of such treatment, a therapeutically effective amount of the compound according to the first aspect or the complex according to the fourth aspect.

It will be appreciated that gene therapy may comprise the administration of nucleic acids (e.g. DNA or RNA) to a subject in need of treatment.
It will be appreciated that the invention may also be used to create a variety of different vectors that can be used for the treatment and/or diagnosis of a variety of diseases depending on the nature of the complex used. For example, in an embodiment where the nucleic acid comprises a tumour-targeting ligand and/or which comprises a transgene expressing an anti-tumour protein (e.g. TNF-alpha), then it may be used to treat cancer. Hence, the target cell in the gene therapy technique is preferably eukaryotic, and preferably mammalian.

It will be appreciated that the PEG amine compound and/or PEG-nucleic acid complex (i.e. “agents”) may be used in a medicament which may be used in a monotherapy, or as an adjunct to, or in combination with, known therapies for treating, ameliorating, or preventing disease, such as cancer. The agents according to the invention may be combined in compositions having a number of different forms depending, in particular, on the manner in which the composition is to be used. Thus, for example, the

composition may be in the form of a powder, tablet, capsule, liquid etc. or any other suitable form that may be administered to a person or animal in need of treatment. It will be appreciated that the vehicle of the medicaments according to the invention should be one which is well-tolerated by the subject to whom it is given.

Medicaments comprising the agents according to the invention (i.e. the complex or aggregate) may be used in a number of ways. For instance, oral administration may be required, in which case the agents may be contained within a composition that may, for example, be ingested orally in the form of a tablet, capsule or liquid. Compositions comprising agents of the invention may be administered by inhalation (e.g. to the lungs or systemically via the lungs) or intranasally to the systemic circulation or intranasally to the brain. Compositions may also be formulated for topical use. For instance as creams, lotions, waxes or ointments may be applied to the skin. Compositions may also be administered as suppositories to the rectum or as pessaries to the vaginal cavity.

Agents according to the invention may also be incorporated within a slow- or delayed-release device. Such devices may, for example, be inserted on or under the skin, and the medicament may be released over weeks or even months. The device may be located at least adjacent the treatment site. Such devices may be particularly advantageous when long-term treatment with agents used according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

In a preferred embodiment, agents and compositions according to the invention may be administered to a subject by injection into the blood stream or directly into a site requiring treatment. Injections may be intravenous (bolus or infusion), intra-arterial (bolus or infusion), subcutaneous (bolus or infusion), intramuscular (bolus or infusion), intrathecal (bolus or infusion), intracerebroventricular (bolus or infusion), intratumoural (bolus or infusion), intraperitoneal (bolus or infusion), or intradermal (bolus or infusion).

It will be appreciated that the amount of the agent that is required is determined by its biological activity and bioavailability, which in turn depends on the mode of administration, the physiochemical properties of the agent, and whether it is being used as a monotherapy, or in a combined therapy. The frequency of administration

will also be influenced by the half-life of the agent within the subject being treated. Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular agent in use, the strength of the pharmaceutical composition, the mode of administration, and the advancement of the disease. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

Generally, a daily dose of between 0.01μg/kg of body weight and 500mg/kg of body weight of the agent according to the invention may be used. More preferably, the daily dose is between 0.01mg/kg of body weight and 400mg/kg of body weight, and more preferably between 0.1mg/kg and 200mg/kg body weight.

The compound or PEG amine - nucleic acid complex may be administered before, during, or after the onset of disease. For example, the compound or PEG amine - nucleic acid complex may be administered immediately after a subject has developed a disease. Daily doses may be given systemically as a single administration (e.g. a single daily injection). Alternatively, the compound or PEG amine - nucleic acid complex may require administration twice or more times during a day. As an example, the compound or PEG amine - nucleic acid complex may be administered as two (or more depending upon the severity of the disease being treated) daily doses of between 25mg and 7000 mg (i.e. assuming a body weight of 70 kg). A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3- or 4-hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses of PEG amine - nucleic acid complex according to the invention to a patient without the need to administer repeated doses.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to form specific formulations comprising the complex or compound according to the invention and precise therapeutic regimes (such as daily doses of the complex or compound and the frequency of administration).

Hence, in an eighth aspect of the invention, there is provided a pharmaceutical composition comprising the compound of the first aspect or the complex of the fourth aspect, and a pharmaceutically acceptable vehicle.

The composition can be used in the therapeutic amelioration, prevention or treatment of any disease in a subject that is treatable with gene therapy, such as cancer.

The invention also provides, in a ninth aspect, a process for making the pharmaceutical composition according to the eighth aspect, the process comprising contacting a therapeutically effective amount of the compound of the first aspect or the complex of the fourth aspect, and a pharmaceutically acceptable vehicle.

A "subject" may be a vertebrate, mammal, or domestic animal. Hence, agents, compositions and medicaments according to the invention may be used to treat any mammal, for example livestock (e.g. a horse), pets, or may be used in other veterinary applications. Most preferably, however, the subject is a human being.

A "therapeutically effective amount" of agent is any amount which, when administered to a subject, is the amount of drug that is needed to treat the target disease in the gene therapy method, or produce the desired effect, e.g. result in tumour killing.

For example, the therapeutically effective amount of agent used may be from about 0.01 mg to about 800 mg, and preferably from about 0.01 mg to about 500 mg.

A "pharmaceutically acceptable vehicle" as referred to herein, is any known compound or combination of known compounds that are known to those skilled in the art to be useful in formulating pharmaceutical compositions.

In one embodiment, the pharmaceutically acceptable vehicle may be a solid, and the composition may be in the form of a powder or tablet. A solid pharmaceutically acceptable vehicle may include one or more substances which may also act as flavouring agents, lubricants, solubilisers, suspending agents, dyes, fillers, glidants, compression aids, inert binders, sweeteners, preservatives, dyes, coatings, or tablet-disintegrating agents. The vehicle may also be an encapsulating material. In powders, the vehicle is a finely divided solid that is in admixture with the finely divided active agents according to the invention. In tablets, the active agent (e.g. the compound or complex of the invention) may be mixed with a vehicle having the necessary compression properties in suitable proportions and compacted in the shape and size

desired. The powders and tablets preferably contain up to 99% of the active agents. Suitable solid vehicles include, for example calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins. In another embodiment, the pharmaceutical vehicle may be a gel and the composition may be in the form of a cream or the like.

However, the pharmaceutical vehicle may be a liquid, and the pharmaceutical composition is in the form of a dispersion. Liquid vehicles are used in preparing solutions, dispersions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The compound or complex of the invention may be dissolved or suspended respectively in a pharmaceutically acceptable liquid vehicle such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid vehicle can contain other suitable pharmaceutical additives such as solubilisers, emulsifiers, buffers, preservatives, sweeteners, flavouring agents, suspending agents, thickening agents, colours, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid vehicles for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the vehicle can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid vehicles are useful in sterile liquid form compositions for parenteral administration. The liquid vehicle for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions, which are sterile solutions or suspensions, can be utilized by, for example, intramuscular, intrathecal, epidural, intraperitoneal, intravenous and particularly subcutaneous injection. The compound or complex of the invention may be prepared as a sterile solid composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium.

The compound or complex of the invention and pharmaceutical compositions of the invention may be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents (for example, enough saline or glucose to make the solution isotonic), bile salts, acacia, gelatin, sorbitan monoleate, polysorbate

80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like. The compound or complex of the invention can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

For a better understanding of the invention, and to show how embodiments of the same may be carried into effect, reference will now be made, by way of example, to the accompanying Figures, in which:-

Figure 1 shows DNA being combined with a first embodiment a PEG amine according to the invention (i.e. 4APEA) to form a PEG amine DNA complex (top panel) and the treatment of an animal tumour with the PEG amine DNA complex (bottom panel);

Figure 2 shows the chemical synthesis of 4APEA from the 4APEG starting material. The global level of additional $-CH_2-CH_2-NH_2$ groups did not exceed 4 per molecule;

Figure 3a shows DNA condensation (mean ± s.d.) by 4APEG and 4APEA, DNA concentration = 0.025 mg mL^{-1};

Figure 3b shows a negative stained transmission electron micrograph of 4APEA – DNA nanoparticles at an N, P ratio of 160 (DNA concentration = 0.250 mg mL^{-1}) in phosphate buffer (2 mM, pH = 6.0), bar = 500 nm;

Figure 3c shows the size (open symbols) and zeta potential (closed symbols) (mean ± s.d.) of 4APEA – DNA nanoparticles prepared in phosphate buffer (2 mM, pH = 6.0). DNA concentration = 0.250 mg mL^{-1}. 4APEG did not condense DNA at any of the nitrogen, phosphate ratios studied;

Figure 4a shows gel electrophoresis data on DNA condensation. The absence of the migration of DNA towards the cathode in the 4APEA–DNA nanoparticles indicates that free DNA was not present in the nanoparticles and the absence of a fluorescent signal for all of the 4APEA–DNA complexes indicates that any DNA contained in the particles was not accessible to the ethidium bromide dye. These two observations provide evidence of DNA condensation by 4APEA. 4APEG did not complex DNA at any of the nitrogen, phosphate ratios studied;

Figure 4b shows gel electrophoresis data on heparin challenge. The 4APEA–DNA complex is resistant to the heparin challenge up to a heparin concentration of 1 mg mL^{-1}. The 4APEG – DNA formulation was susceptible to a heparin challenge at all concentrations tested;

Figure 4c shows gel electrophoresis data on heparin challenge with 4APEA–DNA complexes. The 4APEA–DNA complex was resistant to a challenge with heparin below a heparin concentration of 1 mg mL^{-1};

Figure 4d shows the stability of 4APEA–DNA nanoparticles after incubation for 1 hour in serum and dissociation of the complex using heparin. DNA in all samples remains intact after incubation for 1 h in serum;

Figure 4e shows the stability of 4APEA–DNA nanoparticles after incubation for 2 hour in serum and dissociation of the complex using heparin. There is degradation of the naked DNA and 4APEG–DNA samples after incubation for 2 h in serum. The DNA in the 4APEA – DNA sample remains intact after incubation for 2 h in serum;

Figure 5 shows in vitro transfection efficacy (mean ± s.d.) of the beta galactosidase plasmid using 4APEG–DNA (open bars) and 4APEA–DNA (closed bars) complexes in the A31 cell line. Original cell density = 1000 cells per well, DNA = 1μg per well in 50 μL of medium and incubation time = 4 hours. Transfection is shown relative to transfection with branched PEI [molecular weight = 25 kDa, nitrogen, phosphate (N, P) ratio = 8]. Transfection with naked DNA was 0% ± 1.62 relative to PEI and cells incubated with saline showed a background level of transfection (0.02% ± 1.60) relative to PEI. * = statistically significant difference relative to 4APEG – beta galactosidase gene complexes and all controls ($p < 0.05$);

Figure 6 shows the biodistribution of ^{125}I-TNF-alpha DNA (mean ± s.d.) when injected intravenously as naked DNA (50 μg per mouse, open bars) or as a 4APEA - ^{125}I-TNF-alpha gene complex (5.4 mg and 50 μg per mouse respectively, closed bars). * = statistically significantly different when compared to naked DNA ($p < 0.05$);

Figure 7 shows the tumouricidal activity (mean ± sem) in an MiaPaCa xenograft model. Treated animals received tail vein injections on Days 21, 28, 35, 42, 49 and 56. Groups of animals received either: no treatment (○), the TNF-alpha gene alone (50 μg per mouse, △), the 4APEA - TNF-alpha gene (5.4 mg and 50 μg per mouse respectively ●) and 4APEA (5.4 mg per mouse □). * = statistically significantly different from untreated control animals ($p < 0.05$);

Figure 8 shows the synthesis reaction scheme for a second embodiment a PEG Amine according to the invention (i.e. 4PPA). The global level of $-CH_2-CH_2-CH_2-NH_2$ groups did not exceed 4 per molecule;

Figure 9 shows is a bar chart showing the size of the nanoparticles after 1 hr of complexation of the polymer in a phosphate buffer (2mM, pH = 6.0), with pDNA/ siRNA at a final concentration of 0.025 $mg.mL^{-1}$, 0.020 $mg.mL^{-1}$ respectively. Polydispersity values are shown, as numbers, within the corresponding size column, showing that the nanoparticles were monodisperse;

Figure 10 is a scanning electron microscopy image of the 4PPA – siRNA nanoparticles. Nanoparticles consist of the polymer and siRNA-ITCH at a nitrogen (polymer amine) to phosphate (siRNA) ratio of 60 in phosphate buffer (2mM, pH = 6.0), siRNA concentration = 0.04 $mg\ mL^{-1}$;

Figure 11 shows the in-vitro 6 hr down-regulation in the A431 cell line with a 4PPA – siRNA-ITCH complex (0.010 mg siRNA per well). Lipofectamine complexes with siRNA-ITCH served as a positive control;

Figure 12 are confocal laser scanning micrograph showing cellular uptake of fluorescently labelled siRNA in the cells of the olfactory bulb 1 h after the intranasal administration of: a) 20 μg of Cy3-siRNA-GAPDH as a 4PPA polyplex containing 1.013 mg of 4PPA and within a dose volume of 45 μL and dispersed in phosphate buffer (pH

= 6.0) and b) 20 μg of Cy3-siRNA-GAPDH alone within a dose volume of 20 μL and dissolved in phosphate buffer (pH = 6.0); and

Figure 13 is a Western blotting chromatogram showing the down regulation of the ITCH gene after the intranasal administration of 40 μg siRNA-ITCH twice daily for 3 days as a 4PPA complex at a nitrogen to phosphate ratio of 60 and within a dose volume of 45 μL and dispersed in phosphate buffer. Animals were killed and tissues sampled 18 h after the sixth dose.

Examples

The inventors have prepared various embodiments of a PEG Amine. The first embodiment of PEG Amine was tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(2-ethylamine)-]-pentaerythritol (4-arm-PEG-ethylamine – 4APEA), which is described in Example 1, and the second embodiment of PEG Amine was tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(3-propylamine)-]-pentaerythritol (4PPA), which is described in Example 2.

Example 1 – Preparation and analysis of 4APEA

Referring to Figure 1, the inventors prepared a PEG Amine compound and then combined it with DNA in order to produce a PEG amine - DNA complex. In this example the PEG Amine was tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(2-ethylamine)-]-pentaerythritol (4-arm-PEG-ethylamine – 4APEA). In one embodiment, the DNA used in the complex was the TNF-alpha gene encoding TNF alpha. As described below, and as summarised in the lower panel on Figure 1, on intravenous injection of the 4APEA–TNF-alpha gene complex to a pancreatic cancer xenograft mouse model, the animals dosed with the 4APEA – TNF-alpha gene complex produced a tumouricidal response, with tumour volume significantly decreased relative to untreated controls.

Materials and Methods

Chemicals and reagents were obtained from Sigma Aldrich (UK) unless otherwise stated. Solvents were obtained from Fisher Scientific (UK). All reagents were used without further purification.

Synthesis of tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-N-(2-ethylamine)-]-pentaerythritol (4-arm-PEG-ethylamine – 4APEA)

The synthesis of 4APEA was carried out as shown in Figure 2. Tetra-O,O,O,O-poly(ethyleneglycol-O-2-ethyleneimine) (4APEG, 0.200 g, 0.10 mmol, Jenkem Technology, Inc., USA) was dissolved in Chloroform (10 mL). 2-bromoethylamine hydrobromide (3.28 g, 16.00 mmol) was added to this solution with continuous stirring. Triethylamine (TEA, 10 mL, 7.2 mMoles) was then added to this mixture and the reaction was refluxed at 50°C for 24 hours. At the end of this reaction, the solvent was removed by evaporation under reduced pressure at 50°C. The crude product was collected and dialysed against distilled water over 12 hours (5 L with 6 changes, Visking tubing, molecular weight cut off = 1000 Da). The dialysate was lyophilised and the dried yellow paste was reconstituted in water (1 mL), chromatographed over a Sephadex G-15 gel filtration chromatography column (35 cm packed bed, effective length = 350 mm, diameter = 25 mm) using filtered HPLC water as the eluent. Thirty fractions of 3 mL each were collected and the polymer was recovered in fractions 14 – 18 as a yellowish gel after lyophilisation.

Yield = 0.15g

^{1}H NMR (solvent = D_2O): δ = 2.6 – 3.0 (m, -O-CH_2-***CH_2***-NH-CH_2-CH_2-NH_2 and -O-CH_2-CH_2-NH-***CH_2***-CH_2-NH_2), 3.3 (m, -O-CH_2-CH_2-NH-CH_2-***CH_2***-NH_2), 3.4 – 4.0 (m, -C-***CH_2***-O-CH_2-CH_2-O- and O-***CH_2-CH_2***-O- and -O-***CH_2***-CH_2-NH-CH_2-CH_2-NH_2), 4.7 (water protons).

FTIR v (cm^{-1}): 3400 (N-H stretch, -NH_2), 2869 (C-H saturated stretch, alkane), 1630 (N-H bending, NH_2), 1455 (C-H bending, alkane).

MALDI-TOF: m/z = 2221

Polymer Characterisation

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy experiments were performed on solutions of 4APEG and 4APEA in deuterium oxide (D_2O). All samples were prepared at the concentration of at least 20 mg mL^{-1} to assign non-exchangeable coupled protons (Bruker Aviance 400 MHz spectrometer, Bruker Instruments, UK).

Fourier transform infra-red (FTIR) spectroscopy analyses were carried out on a Perkin Elmer Precisely Spectrum 100: FTIR spectrometer (Perkin Elmer, UK). The experiments were performed with polymer samples (~ 2 mg) at a wavenumber range of 4000 cm^{-1} to 40 cm^{-1} with a resolution of 4 cm^{-1} in the absorbance mode at room temperature.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometery (MALDI-TOF-MS) experiments were carried out on a Voyager-DE PRO Biospectrometry Workstation (PerSeptive Biosystems, Inc., USA). During the preparation, 2,5-dihydroxy benzoic acid (10 mg mL^{-1}) in acetonitrile, water, trifluoroacetic acid (50: 50: 0.1) was used as a matrix and mixed at a 1: 1 weight ratio with the sample (4APEA or 4APEG). Data were collected and processed using the Voyager Biospectrometry workstation.

Preparation of DNA Formulations

DNA Production

Plasmids expressing the pCMV-β-galactose gene were propagated in *E. Coli*. The *E.coli* DH5-alpha strain was used to produce plasmid DNA. Briefly, Luria Broth Base (25 g L^{-1}, 2L) in MilliQ water was autoclaved. Using aseptic techniques, Ampicillin (50 μg mL^{-1}) was supplemented in the medium at room temperature and an aliquot of the solution (2 mL) was transferred to a 25 mL centrifuge tube. An aliquot of the seed culture of E. coli DH5-alpha strain, sufficient to provide a suitable level of colonies, was transferred to the centrifuge tube and incubated at 37°C for 4 h. Subsequently the whole bacterial solution from the centrifuge tube was added to the autoclaved medium culture (2 L) and fermented at 37°C for 16 h with shaking at 120 rpm. The bacterial cells were harvested by centrifugation at 6000 rpm at 4°C for 15 min (Hermle Z323K, Germany). The supernatant was removed and the pellet was processed according to the manufacturer's instruction (Qiagen EndoFree Plasmid Giga Kit, Qiagen, UK). The DNA solution in MilliQ water was desalted through the illustra NAP™ columns (GE Healthcare life Sciences, UK). The DNA concentration in the final solution was measured with a UV-VIS spectrophotometer (Nanodrop2000 UV-Vis Spectrophotometer, Thermo Scientific, UK) at a wavelength of 260 nm and the purity of the DNA estimated by measuring the relative absorbance (260/ 280 nm). The concentration range of the final solutions was 0.5 - 1 mg mL^{-1} and the total yield of the extracted plasmid DNA was in range of 0.5 - 1.0 mg for each batch.

Polyplex Production

Polyplexes were produced using: 4APEG and 4APEA. Polyplexes were prepared in sterile phosphate buffer [Sodium phosphate dibasic (Na_2HPO_4, 0.2 M) and Sodium dihydrogen phosphate (NaH_2PO_4, 0.2 M), diluted to 100 mL to give 2mM phosphate buffer, pH = 6.0] to a final DNA concentration of 250 μg mL^{-1}. To the DNA solutions (0.1 mL, pDNA expressing the -β-galactose gene under the control of the cytomegalovirus promoter) in sterile phosphate buffer (pH = 6.0) were added equal volumes of the polymer stock solutions in phosphate buffer (pH = 6.0) at various nitrogen (polymer), phosphate (DNA) ratios (N, P ratios). The complexes were gently mixed using a pipette and allowed to incubate at room temperature for 15 minutes before use. The resulting complexes were sized and their zeta potential measured (Malvern Zetasizer 3000HS, UK).

The complexes were also imaged using transmission electron microscopy with negative staining (1% aqueous uranyl acetate solution) after application to carbon-coated 200 mesh copper grids. The complex formation was analysed using an agarose gel (0.7 % w/w) in a Tris-acetate-EDTA buffer chamber. The current (PowerPac ™, Bio-Rad Laboratories Ltd., UK) was applied for 1 hr at 90 V. The results were imaged on a UV Transilluminator (Gene snap, Syngene, UK). The relative level of unbound DNA in the complexes was investigated using the fluorescent ethidium bromide displacement assay [12] and an LS50B spectrofluorometer (Perkin Elmer, Cambridge, UK).

Complex Stability and Biocompatibility

Heparin Stability

The experimental procedure was modified from a reported method (Liu, Y.; Reineke, T. M., Poly(glycoamidoamine)s for gene delivery: stability of polyplexes and efficacy with cardiomyoblast cells. *Bioconjug. Chem.* 2006, 17, (1), 101-8). The polymer solution in phosphate buffer (pH = 6.0, 5 μL) and the pDNA solution (1 μg in 10 μL) at an N, P ratio of 80 were mixed and incubated for 15 min at room temperature. Stock heparin was initially prepared at 5000 μg mL^{-1} and diluted to make 100 - 1000 μg mL^{-1} heparin solutions. Heparin (at various concentrations, 10 μL) was added to the complex dispersion (10 μL) and the dispersion incubated for 15 min. The dispersion was then subjected to gel electrophoresis for 1 hr at 90 V in order to investigate complex stability on challenge with an anionic polyelectrolyte.

Stability in Serum

Aliquots of the polymer solution in phosphate buffer (2 mM, pH = 6.0, 5 μL) and the pDNA solution (100 μg mL^{-1}, 10 μL) in phosphate buffer (2 mM, pH = 6.0, 5 μL) were mixed at an N, P ratio of 80 and incubated for 15 min at room temperature. Foetal bovine serum (FBS, 5 μL) was added to the complex and the mixture was incubated at 37°C and samples withdrawn at the 1 and 2 h time points. Ethylene diamine tetra acetic acid (EDTA, 250 mM, 5 μL) was added to deactivate nucleases and the serum – polyplex mixture incubated for a further 10 mins. Subsequently, heparin solution (5 mg mL^{-1}, 10 μL) was added to allow complex dissociation and the mixture was then incubated for a further 2 h. The complex dispersion was subjected to gel electrophoresis for 1 hr at 90 V to investigate the stability of the complex in the presence of serum nucleases. Samples containing only pDNA were used as controls.

Samples of 4APEA – DNA (DNA concentration = 0.2 mg mL-1, 5 μL) were added to 50 %v/v serum in phosphate buffer (2 mM, pH = 6.0) vehicle and incubated at 37 °C. At the 1 and 2 hour time points, samples were removed and sized using the Malvern Zeta Sizer after diluting 5 times with phosphate buffer (2 mM, pH = 6.0).

Cell cytotoxicity

Substance cytotoxity was assessed by the measurement of the IC_{50} value in a standard MTT assay. This assay is based on an ability of living cells to transform a water-soluble yellow dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide - MTT) into purple coloured water-insoluble formazan by cellular mitochondrial succinate dehydrogenase (Shau, M. D.; Shih, M. F.; Lin, C. C.; Chuang, I. C.; Hung, W. C.; Hennink, W. E.; Cherng, J. Y., A one-step process in preparation of cationic nanoparticles with poly(lactide-co-glycolide)-containing polyethylenimine gives efficient gene delivery. *Eur. J. Pharm. Sci.* 2012, 46, (5), 522-529). The experimental procedure was derived from a reported method (Uchegbu, I. F.; Sadiq, L.; Arastoo, M.; Gray, A. I.; Wang, W.; Waigh, R. D.; Schatzlein, A. G., Quaternary ammonium palmitoyl glycol chitosan--a new polysoap for drug delivery. *Int. J. Pharm.* 2001, 224, (1-2), 185-199).

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide blue-indicator dye) assay used in this study was developed to evaluate the cytotoxicity. The endpoint is based on how many cells were being killed. As counting cells is inapplicable, the inventors used this assay which measures the amount/activity of a cellular enzyme.

The results can be read on a multi-well scanning spectrophotometer and shows a high degree of precision. No washing steps were used in the assay. The main advantages of the colorimetric assay are its rapidity and precision, and the lack of any radioisotope contamination.

A human epidermoid carcinoma cell line (A431, ATCC CR L-1555) was maintained in Minimum essential medium (MEM) supplemented with FBS (10% v/v) and glutamine (2 mM) in 10% CO_2 at 37°C. Briefly, 96-well microtitre plates were seeded with 5000 cells per well and incubated for 72 h. A solution of the polymers (15 mg mL^{-1}) was prepared in phosphate buffer (2 mM, pH = 6.0) and serial dilutions of the polymer in tissue culture media were incubated with the cells for 4 h. The polymer test solution was then replaced with fresh medium. Subsequently the medium was changed daily and the cells incubated for a further 72 h. After which the MTT solution (50 µL, 0.5 mg mL^{-1} in fresh medium) was added to each well. After incubation for 4 h in the dark, the medium and MTT solution were removed and the cells were lysed with dimethylsulphoxide (DMSO, 200 µL). The absorbance of each well was measured at 570 nm. Values were expressed as a percentage of the control to which no polymer was added. Triton X-100 (1% w/v) was used as a positive control and untreated cells as the negative control.

In vitro Cell transfection efficiency

Briefly, 96-well microtitre plates were seeded with 10,000 cells per well and incubated overnight. Suspensions of the polymer – gene complex (N, P ratios of 1, 5, 40, 80 and 160) were prepared in phosphate buffer (2 mM, pH 6.0, 50 µL) and incubated for 15 minutes. The medium (DMEM) was removed from the cells prior to the study and the cells were washed twice with PBS. Fresh DMEM (150 µL) was added to the cells, followed by addition of the polymer – gene complex dispersion (50 µL) and the cells incubated for 4 h. The test samples were then replaced with fresh MEM and the MEM refreshed daily while the cells were incubated for a further 48 h. The cells were lysed with triton X-100 (2% w/v, 50 µL) and kept at -80°C for at least 15 min. After defrosting, inactivated FBS (0.5 % v/v, 50 µL in PBS) was added to make the total volume to 200 µL. O-Nitrophenyl ß-D-Galactopyranoside solution (ONPG, 2 mg mL^{-1}, 100 µL) was transferred to each well and the mixture incubated for 2 h. Subsequently the absorption was measured at 420 nm. Values were expressed as a percentage of the absorbance observed with the positive control (branched PEI) and compared to the data observed with the naked plasmid DNA and untreated samples.

In vivo bio-distribution studies

TNF-alpha DNA was obtained as previously described. The plasmid was radiolabelled using IODO-GEN (Fisher Scientific, UK). In an IODO-GEN tube (Pierce Pre-Coated Iodination Tubes, 12 X 75mm, Fisher Scientific Pierce, UK), TNF-alpha plasmid (1.0 mg mL^{-1}, 10 μL) Sodium acetate (0.1 M, 5.25 μL, pH = 4) and ^{125}I-Sodium iodide (10 mCi mL^{-1}, 5 μL) were added. The mixture was incubated at 50°C for 30 min. After chilling on ice, NaOH (0.1 M, 5 μL) was added to stop the reaction. Excess iodine was removed using a Bio-Spin 30 column. The Bio-Spin 30 column (Bio-Rad Laboratories, Lifescience, UK) was inverted sharply several times to re-suspend the settled gel and remove the air bubbles. The tip was removed and the column was placed in a 2.0 mL spin column-wash microcentrifuge tube. The cap was also removed to allow the excess packing buffer to drain. The drained buffer was discarded and the column was placed back into the 2.0 mL tube to be centrifuged (2 min at 1000g) in order to remove the remaining packing buffer. The drained Bio-Spin 30 column was placed in a clean 1.5 mL microcentrifuge collection tube. The solution from the IODO-GEN tube was gently applied to the top of the gel bed and the column was centrifuged for 4 min at 1000g (Mini Centrifuge, Bio-Rad Laboratories, Lifescience, UK). The purified sample was diluted 1 in 100 with unlabelled TNF-alpha DNA and made up to 1 mL with filtered phosphate buffer (pH = 6.0). The solution of ^{125}I-TNF-alpha DNA (250 μg mL^{-1}) and the ^{125}I-TNF-alpha DNA – 4APEA nanoparticles were prepared in sterile phosphate buffer (2 mM, pH 6.0) as described above. The ^{125}I-TNF-alpha DNA – 4APEA nanoparticles were prepared at a DNA concentration of 250 μg mL-1 and a 4APEA concentration of 26.7 mg mL^{-1}.

The MiaPaCa cell line (ATCC) was maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with FBS (10 % v/v) and L-glutamine (2 mM) in 10% CO_2 at 37 °C. A suspension of the cells (5,000,000 cells in 0.1 mL) was prepared in cell culture medium and Matrigel® at a 1: 1 volume ratio and the cell suspension (100 μL) was subcutaneously injected in the left flank of CD-1 female nude mice (16 - 20 g). When the tumour reached approximately 50 - 100 mm^3 in size, the CD-1 female nude mice were dosed with either a solution of ^{125}I-TNF-alpha DNA alone (250 μg mL^{-1}, 200 μL) or4APEA - ^{125}I-TNF-alpha DNA nanoparticles (^{125}I-TNF-alpha DNA = 250 μg mL^{-1}, 4APEA = 26.7 mg mL^{-1}, 200 μL). At two time points (15 and 60 minutes), animals were killed organs sampled and radioactivity measured using a gamma counter (2470 Perkin Elmer Automatic Gamma counter Wizard², UK).

In vivo tumour regression studies

A batch of pORF 9-mTNF-alpha was produced as described above under DNA Production. The yield was 1 – 1.2 mg plasmid DNA per batch. The MiaPaCa cell line (ATCC) was maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with FBS (10 % v/v) and L-glutamine (2 mM) in 10% CO_2 at 37 °C. A suspension of the cells (5,000,000 cells in 0.1 mL) was prepared in cell culture medium and Matrigel® at a 1: 1 volume ratio and the cell suspension (100 µL) was subcutaneously injected in the left flank of CD-1 female nude mice (16 - 20 g). When the tumour reached approximately 50 - 100 mm^3 in size, the CD-1 nude mice were grouped and dosed with different DNA (pORF 9-mTNF-alpha) or control formulations.

Groups (n = 5) of CD-1 female tumour bearing mice (16 - 20 g) were intravenously administered via a tail vein injection with: a) the TNF-alpha DNA solution (250 µg mL^{-1}, 200 µL) in phosphate buffer (2 mM, pH = 6.0), b) a 4APEA solution (27 mg mL^{-1}, 200 µL) in phosphate buffer (2 mM, pH = 6.0), c) the 4APEA – TNF-alpha DNA nanoparticles (TNF-alpha DNA = 250 µg mL^{-1}, 4APEA = 27 mg mL^{-1}, 200 µL) in phosphate buffer (2 mM, pH = 6.0) or d) left as an untreated controls. Treated animals were dosed weekly for a maximum of 6 consecutive weeks. Body weight, tumour volume and animal behaviour were monitored.

Statistical analysis

Statistical analyses were performed using One-Way ANOVA, followed by post tests. A p-value of < 0.05 was considered to be significant. Analysis was performed using Minitab software (Version 15.1.30.0, 2007 Minitab Inc, Coventry, UK).

Experiment A - Polymer Synthesis

Tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(2-ethylamine)-]-pentaerythritol (4-arm-PEG-ethylamine – 4APEA) was prepared in good yield using the chemical synthesis route shown in Figure 2, and then characterised. Using the MALDI-TOF data for the parent 4PEGA (m/z = 2049.3) and for the 4APEA (m/z = 2221), the level of ethylamine substitution on the parent 4PEGA compound was 100%. In comparison to 4APEG, there was a global level of 4 additional ethylamine moieties per each 4APEA molecule. The presence of $–NH-(CH_2-CH_2-NH)_n-CH_2-CH_2-NH_2$ groups could not be ruled out.

Experiment B – Polyplex Production

4APEA was found to condense DNA at fairly high N, P ratios (> 40: 1 - Figure 3a and 4a) and to form 200 nm (Figure 3b and 3c), positively charged (Figure 3c) polyplexes with DNA. The starting PEG amine, 4APEG did not condense DNA at any of the nitrogen to phosphate ratios studied (Figure 3a, and Figure 4a). It is clear that adding at least 4 additional terminal amines per molecule to 4APEG converted 4APEG into a DNA condensing molecule.

Experiment C - Polyplex Stability

In order to investigate the stability of the polyplexes in the presence of endogenous biopolymers such as proteins and polyanions, the polyplexes were challenged with heparin (Figure 4b and 4c) and serum (Figure 4d and 4e). Uncondensed DNA is seen in all 4APEG – DNA mixtures in the presence of heparin, while condensed DNA is seen with the 4APEA – DNA nanoparticles in the presence of heparin up to a heparin concentration of 1 mg mL^{-1}. Above this concentration of 1 mg mL^{-1} heparin, DNA is dissociated from the 4APEA - DNA complex and appears as uncondensed DNA (Figure 4c). In serum, after 1h there is surprisingly no degradation of DNA detected with the gel electrophoresis analytical technique (Figure 4d). After 2h however there is marked degradation of naked DNA, which is prevented by DNA complexation with 4APEA (Figure 4e). 4APEG is ineffective in the protection of DNA from degradation by serum nucleases at the 2h time point (Figure 4e), presumably because this polymer does not condense DNA (Figure 4a). On incubation with serum there was a change in 4APEA – DNA complex size after incubation for 1h in serum, and the surface charge became neutral, presumably after being coated with serum proteins (Table 1). It is clear from this data that adding additional amines to 4APEG enables DNA condensation and the formation of DNA nanoparticles; this in turn protects the DNA from degradation. Additionally, the inventors also observe that the complexes attain a neutral zeta potential on incubation with serum proteins and that this does not lead to aggregation of the complexes *in vitro* (Table 1).

Table 1 – 4APEA – DNA (N, P Ratio = 80) Complex Stability in Serum

Time after incubation in plasma (50 % v/v) in phosphate buffer (2 mM, pH = 6.0) (mins).	0	15	60
Z-average mean diameter (mean ±	193 ± 1.94	39 ± 16	32 ± 3

Time after incubation in plasma (50 % v/v) in phosphate buffer (2 mM, pH = 6.0) (mins).	0	15	60
s.d. nm)			
Polydispersity (mean ± s.d.)	0.42 ± 0.01	0.35 ± 0.10	0.25 ± 0.01
Zeta potential (mean ± s.d. mv)	+20 ± 1.70	-3.5 ± 2.0	-3.1 ± 1.9

Experiment D – *In Vitro* Cell Cytotoxicity

Table 2: Cytototoxicity in the A431 cell line

Compound/ Formulation	IC50 (mean ± s.d. mg mL^{-1})
4APEG	2.70 ± 0.14
4APEA	4.76 ± 0.27
4APEA – DNA (N, P ratio = 20)	6.36± 0.16

The PEG compounds were relatively non-toxic to the A431 cell line (Table 2), with IC50 values in the mg mL^{-1} (millimolar) range. Crucially the addition of additional amine groups did not increase the cytotoxicity of 4APEG and A431 cell cytotoxity IC50 values for 4APEA and its DNA complex were similar to those observed for chitosan derivatives (0.7 – 1.3 mg mL^{-1}) in Uchegbu, I.F. Sadiq, L., Pardakhty, A., El-Hammadi, M., Gray, A.I., Tetley, L., Wang, w., Zinselmeyer, B.H., Schatzlein, A.G. Gene transfer with three amphiphilic glycol chitosans - the degree of polymerisation is the main controller of transfection efficiency, J. Drug Target. 2004, 12, 527-539. and were over 1000 times higher than the values reported by the inventors for PEI in the same cell line (0.0019 – 0.0036 mg mL^{-1}) in Brownlie, A.; Uchegbu, I. F.; Schatzlein, A. G., PEI-based vesicle-polymer hybrid gene delivery system with improved biocompatibility. *Int. J. Pharm.* 2004, 274, (1-2), 41-52.

Experiment E – *In Vitro* Cell Transfection

These 4APEA complexes were also able to transfer DNA into cells and as evidenced by the protein expression measured. It may be concluded that these complexes were also able to transfer DNA to the nucleus with a similar efficacy to PEI (Figure 5).

Surprisingly, 4APEG, despite being unable to condense DNA (Figure 3a) is still able to transfect cells *in vitro* (Figure 5). *In vivo* this agent is not expected to be an efficient gene transfer agent as it would not be able to protect DNA from degradation in the

serum (Figures 4d and e). The inventors thus conclude that a relatively low amine group density, in a gene transfer agent, facilitates the delivery of genes into cells, while the protection of DNA from serum degradation requires a higher amine density in the gene delivery molecule.

The combination of the very good cell biocompatibility and transfection efficacy already proves the working hypothesis in that DNA vectors may be prepared from molecules with a reduced amine content when compared to materials such as PEI and the poly(propylenimine) dendrimers, where one out of every 2 and 3 main chain atoms, respectively, is an amine nitrogen in the latter two molecules, whereas in the present 4APEA vector, approximately one main chain atom out of every eighteen main chain atoms is an amine nitrogen.

Experiment F - *In Vivo* Biodistribution and Activity of 4APEA Complexes

DNA biodistribution could only be investigated in the first 60 minutes after dosing (Figure 6), as DNA was only stable for 1h in serum (Figures 4d and 4e). On intravenous administration, complexed DNA was more rapidly cleared from the blood (Figure 6a) and was distributed to the lungs and liver (Figures 6c and 6f), while free DNA was cleared by the kidneys (Figure 6d). Splenic distribution was similar for both complexed and free DNA (Figure 6e). Distribution to the tumour was doubled at the 60 minute time point when DNA was in the form of a complex (Figure 6b), although the difference was not statistically significant at this time point. These data indicate that while uncomplexed DNA is rapidly cleared by the kidneys, complexed DNA distributes quite early to the first vascular bed encountered – the lung tissue and then particles accumulate more slowly in the liver (Figure 6). It was not possible to observe, within the time window examined, a significantly increased distribution to the tumour tissue although there was a trend towards increased tumour accumulation at the 60 minute time point. The short 4APEA – DNA complex residence time in the blood may have contributed to the relatively poor distribution to the tumour tissue.

Experiment G - *In Vivo* Tumouricidal Activity of 4APEA – DNA Complexes

On intravenous injection of the 4APEA – TNF-alpha gene complex to a pancreatic cancer xenograft mouse model, the animals dosed with the 4APEA – TNF-alpha gene complex produced a tumouricidal response, with tumour volume significantly decreased relative to untreated controls (Figure 7). The TNF-alpha gene alone did not produce a tumour response with one animal having to be culled on Day 50 due to

excessive tumour growth. 4APEA showed a trend towards a tumouristatic response although tumour volumes were not significantly different from untreated controls. There were no significant differences in animal weight when the treatment groups were compared to the control groups (data not shown).

Example 2 – Preparation and analysis of 4PPA

Following on from the experiments carried out in Example 1, the inventors then prepared a second embodiment of a PEG Amine, i.e. tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(3-propylamine)-]-pentaerythritol (4PPA).

Materials and Methods

Chemicals and reagents were obtained from Sigma Aldrich (UK) unless otherwise stated. Solvents were obtained from Fisher Scientific (UK). All reagents were used without further purification.

Synthesis of tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-N-(3-propylamine)-]-pentaerythritol (4PPA)

With regard to the synthesis as shown in Figure 8, 4APEG (0.101 g, 0.05 mmoles) was dissolved in chloroform (10mL). Then, 3-(Boc-amino)propyl bromide (0.1905 g, 0.8 mmoles) was added to the 4APEG solution with continuous stirring. To the reaction was then added triethylamine (10 mL). The pH of the resulting reaction solution was pH = 9 – 10. The reaction was then transferred to a 50 mL flask and refluxed for 24 hours at 50°C with continuous stirring. Following the 24 hours reflux, the reaction solvent was removed by evaporation under reduced pressure at 50 °C. Into the same solid like paste of the boc-amino protected polymer was added drop wise, a HCl solution (3M, 20mL, VWR Ltd., UK) . The resulting solution was left stirring for 90 minutes in order to deprotect all of the boc-amino groups. The resulting de-protected amine solution was subsequently dialyzed using a dialysis membrane (Spectrum Laboratories, Inc., USA, molecular weight cut off = 1.0 kDa) against water (5 L) over 24 hours with 5 changes of the dialysis medium. The dialysate was lyophilized and the product was obtained as a pale yellow gel-like material. The lyophilized polymer was further purified by gel filtration chromatography using a Sephadex G-25 gel filtration chromatography column (30 cm packed bed, effective length = 300 mm, diameter = 18 mm) and eluted with Milli-Q water. Forty 3 mL fractions were collected and the

polymer was recovered in fractions 3 – 15 after lyophilisation. 4PPA presented as a yellow gel like semisolid.
Yield = 0.04g

^{1}H NMR (solvent = D2O): δ = 2.1 (m, -NH-CH_2-**CH_2**-CH_2-NH_2), 3.1 (t, -NH-**CH_2**-CH_2-CH_2-NH_2), 3.2 (m, -NH-CH_2-CH_2-**CH_2**-NH_2), 3.3 (m, -O-CH_2-**CH_2**-NH-CH_2-CH_2-CH_2-NH_2), 3.5 (s, C-**CH_2**-O-), 3.6 – 4.0 (b, -O-**CH_2**-CH_2-NH_2-CH_2-CH_2-CH_2-NH_2, and -O–**CH2-CH2**-O-).

FTIR v (cm -1): 3464 (N-H stretch, -NH_2), 2869 (C-H saturated stretch, alkane), 1638 (N-H bending, NH_2), 1459 (C-H bending, alkane).

MALDI-TOF: m/z= 2287 Da

Polymer Characterisation

Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy experiments were performed on solutions of 4APEG and 4APPA in deuterium oxide (D_2O). All samples were prepared at the concentration of at least 20 mg mL^{-1} to assign non-exchangeable coupled protons (Bruker Aviance 400 MHz spectrometer, Bruker Instruments, UK).

Fourier transform infra-red (FTIR) spectroscopy analyses were carried out on a Perkin Elmer Precisely Spectrum 100: FTIR spectrometer (Perkin Elmer, UK). The experiments were performed with polymer samples (~ 2 mg) at a wavenumber range of 4000 cm^{-1} to 40 cm^{-1} with a resolution of 4 cm^{-1} in the absorbance mode at room temperature.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometery (MALDI-TOF-MS) experiments were carried out on a Voyager-DE PRO Biospectrometry Workstation (PerSeptive Biosystems, Inc., MA, USA). During the preparation, 2,5-dihydroxy benzoic acid (10 mg mL^{-1}) in acetonitrile, water, trifluoroacetic acid (50: 50: 0.1) was used as a matrix and mixed at a 1: 1 weight ratio with the sample (4APPA or 4APEG). Data were collected and processed using the Voyager Biospectrometry workstation.

Polyplex Production

Gene Polyplexes

DNA was obtained as detailed in Example 1. DNA polyplexes were produced using 4PPA. Polyplexes were prepared in sterile dextrose (5% w/v) to a final DNA concentration of 20 μg mL^{-1}. The polyplexes were prepared by adding DNA solutions (0.5 mg mL^{-1}, 0.025 mL, pDNA expressing the -β-galactose gene under the control of the cytomegalovirus promoter) in sterile dextrose (5% w/v) to appropriate volumes of the polymer stock solution in sterile dextrose (5% w/v), such that polyplexes were prepared at nitrogen (polymer), phosphate (DNA) ratios of 1, 4, 8, 16, 32, 40, 60, 80 and 100. The complexes were gently mixed using a pipette and allowed to incubate at room temperature for between 1 – 3 hours before use. The resulting complexes were sized and their zeta potential measured (Malvern Zetasizer 3000HS, Malvern, UK).

Short interference RNA (siRNA) Polyplexes

siRNA polyplexes were produced using 4PPA. Polyplexes were prepared in sterile phosphate buffer (2 mM, pH = 6) to a final siRNA concentration of 20 μg mL^{-1}. The polyplexes were prepared by adding siRNA solutions [1 mg mL^{-1}, 0.01 mL, siRNA-ITCH (SEQ ID No:1)] in sterile phosphate buffer (2mM, pH = 6.0) to appropriate volumes of the polymer stock solution in sterile phosphate buffer (2 mM, pH = 6.0), such that polyplexes were prepared at nitrogen (polymer), phosphate (siRNA) ratios of 1, 4, 40, 60, 80, 100 and 200. The complexes were gently mixed using a pipette and allowed to incubate at room temperature for 1 hour before use. The resulting complexes were sized and their zeta potential measured (Malvern Zetasizer 3000HS, Malvern, UK).

In Vitro Cytotoxicity

The aim of this assay was to study the cytotoxic effect of the polymer itself and to benchmark the polymer's cytotoxicity against any commercially available delivery agents. Safety is one of the main features that the invention offers, and so this assay was important to validate if there are any advantages over the control delivery systems. In addition, a cytotoxicity study would provide a preliminary insight into the behaviour of the delivery vector when it is introduced systemically, i.e. within the physiological conditions. As different formulations vary hugely in this respect the measure that is being used to compare them is the half maximal inhibitory concentration (IC50). IC50 is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure illustrates the half maximal (50%) inhibitory

concentration (IC) of the polymer over a given time period. According to the FDA, IC50 represents the concentration of a substance (i.e. the 4PPA polymer in this context) that is required for 50% inhibition of an effect on the cells in-vitro.
Furthermore, to know whether a formulation is potentially toxic and to find out at which concentration it should be administered in-vivo, this assay was initiated particularly to identify the IC50. There are several methods to identify the IC50. In this study, the MTT assay was used. The section below describes the method applied to determine the IC50 using the MTT assay.

The cell lines used in the MTT assay were similar to those used in the *in-vitro* transfection assay. A human pancreatic carcinoma cell of (MIA PaCa-2, ATCC CR L-1420) and the human epidermoid carcinoma cell line (A-431, ATCC CR L-1555) where both used as cell lines in this assay. MIA PaCa-2 was maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with foetal bovine serum (FBS) (10% v/v) and glutamine (2mM) in 10% CO_2 at 37 °C. A-431 cells were maintained in Modified Eagle Medium (MEM) supplemented with foetal bovine serum (FBS) (10% v/v) and glutamine (2mM) in 10% CO_2 at 37 °C. The cytotoxicity of 4PPA was assessed by the measurement of the IC50 value in a standard MTT assay as described above. 96-well microtiter plates were seeded with 500 or 5000 cells per well of MIA PaCa-2 or A-431 cells respectively and incubated for 72 hr with daily medium changes. A solution of the polymer (100 mg mL^{-1}) was prepared in phosphate buffer (2 mM, pH = 6.0) and serial dilutions of the polymer solution (X µL) were added to each well. To each well was added Dulbecco's Minimum Essential Medium (DMEM) devoid of foetal bovine serum (50 µL). The cells were incubated for 6 hr at 37°C . At the end of the incubation time, the cells were washed twice with phosphate buffered saline (PBS, pH = 7.4) to ensure all of the polymer solution had been removed. The cells were then replenished with samples fresh medium and incubated for a further 48 hr post-treatment, with daily changes of the medium. Cells were then washed twice with PBS and to the each well was added the MTT reagent (0.5 mg mL^{-1}, 50 µL,) in fresh medium. After incubation for 3 hr at 37°C,, the medium and MTT solution were removed, the cells were lysed with dimethylsulphoxide (DMSO) (200 µL) and the absorption measured at 570 nm. Triton-X 100 was used as a positive control, while cells without the polymer treatment were used as a negative control. Values were expressed as a percentage of the control to which no polymer was added.

In-vitro pDNA Transfection

Transfection is the process of deliberately introducing a nucleic acid into cells. The term is used notably for non-viral methods of delivering nucleic acids into eukaryotic cells. In this set of experiments, transfection was the definitive proof of concept for the utility of 4PPA to deliver nucleic acids into cells and transfection was demonstrated by the delivery of the beta-galactosidase gene into cells. Transfection was examined in a human epidermoid carcinoma cell line (A-431), which was obtained from the ATCC with a passage number of 1, and a reference number (CRL-1555™).

Cells (A-431) were cultured in MEM. Cultured cells were in 75-cm^2 flasks maintained at 37 °C and 5% CO_2. Upon reaching confluence, the cells were passaged by aspirating off the media, washing with PBS - EDTA (pH = 7.4, 10 mL), adding trypsin - EDTA (0.25% w/v trypsin, 3 mL) and then incubating with the same volume (3 mL) of trypsin - EDTA (0.25%w/v trypsin) for 3 minutes. Trypsinisation was stopped by the addition of complete medium (7 mL), and the cells were centrifuged (3,000 rpm for 3 min). Cells were re-suspended in complete medium (10 mL) and an aliquot of this suspension (3 mL) seeded. The medium was changed every other day.

Ninety-six-well microtitre plates were seeded with 10,000 cells per well and incubated overnight. Suspensions of the polymer – gene complex (N, P ratios of 40, 60, 80 and 100) were prepared in phosphate buffer (2 mM, pH 6.0, 50 μL) and incubated for 1 hour. The medium (DMEM) was removed from the cells prior to the study and fresh DMEM (150 μL) was added to the cells, followed by addition of the polymer – gene complex dispersion (50 μL containing 1μg of DNA) and the cells incubated for 4 h and 24 h. A Lipofectamine was used as a positive control and formulated according to the manufacturer's instructions (Invitrogen Life Technologies, UK) and was dosed to the cells at a DNA concentration of 0.1 μg per well. Additionally a Generation 3 poly(propylenimine) dendrimer (DAB 16) was also used as a positive control at a nitrogen to phosphate ratio of 5 and 8 (Zinselmeyer B.H., S.P. Mackay, A.G. Schatzlein, I.F. Uchegbu, The lower-generation polypropylenimine dendrimers are effective gene-transfer agents, Pharm. Res., 19, 2002, 960-967.) DAB 16 is a well known transfecting polymer. Untreated cells were used as the negative control. Following the incubation period, the medium was removed and the cells were washed with PBS (pH = 7.4). Fresh medium (MEM) was added and the MEM refreshed daily while the cells were incubated for a further 48 h.

In order to measure the expression of the ß -galactosidase gene upon transfection with the 4PPA polyplexes, the inventors needed an assay to reveal level of the expressed protein, i.e. the ß -galactosidase enzyme. As they were inspecting an enzyme, they needed to supply the enzyme with a substrate of interest that the enzyme can process into a detectable marker. This substrate was O-Nitrophenyl ß-D-Galactopyranoside (ONPG).

The cells were lysed with triton X-100 (2% w/v, 50 µL) and kept at -80°C for at least 15 min. After defrosting, inactivated FBS (0.5 % v/v, 50 µL in PBS) was added to make the total volume to 200 µL. An aliquot of ONPG solution (2 mg mL^{-1}, 100 µL) was transferred to each well and the mixture incubated for 2 h. Subsequently the absorption was measured at 420 nm (ELx808 Absorbance Microplate Reader, BioTek® Instrument INC, USA) and the transfection activity expressed as mU per microgram of plasmid supplied to each well.

In-vitro siRNA mediated down-regulation

In this set of experiments, transfection with siRNA served as another proof of concept for the delivery of nucleic acids to cells by the 4PPA polyplexes. siRNA-ITCH was used to illustrate this delivery capability. Transfection was examined on the human epidermoid carcinoma cell line (A-431), which was obtained from the ATCC with a passage number of 1, and a reference number (CRL-1555™). A-431 only was chosen as a cell line of interest for ITCH down-regulation due to the abundance of expression of itchy E3 ubiquitin protein ligase (ITCH) within the genome of this cell line.

Cells (A-431) were cultured in MEM and cultured cells were in 75-cm^2 flasks maintained at 37 °C and 5% CO_2. Upon reaching confluence, the cells were passaged by aspirating off the media, washing with PBS - EDTA (pH = 7.4, 0.46 mg mL^{-1} EDTA, 10 mL), adding trypsin - EDTA (0.25% w/v trypsin, 3 mL) and then incubating with the same volume (3 mL) of trypsin - EDTA (0.25 % w/v trypsin and 0.38 mg mL^{-1} EDTA) for 3 minutes. Trypsinization was stopped by the addition of complete medium (7 mL), and the cells were centrifuged (3,000 rpm for 3 min). Cells were re-suspended in complete medium (10 mL) and an aliquot of this suspension (3 mL) seeded. The medium was changed every other day.

For transfection, a confluent flask was passaged approximately for 5 passage numbers, and then T25cm flasks were seeded with 400,000 cells per flask and incubated

overnight. Suspensions of the complex between the 4PPA and siRNA-ITCH complexes (N/P ratio of 40, 60, 80 and 100) were prepared in phosphate buffer (2mM, pH 6) and in a final volume of 500 μL (containing 10μg siRNA-ITCH). The resulting polyplex dispersion (500 μL containing 10μg siRNA-ITCH) was then added to the cells and the cells incubated for 6 hrs. Lipofectamine® 2000 (30 μL) was complexed with siRNA-ITCH at a final siRNA-ITCH dose of (3 μg) as per the manufacturer's protocol and used as the positive control. Non treated cells and 4PPA – scrambled siRNA were used as negative controls.

At the end of the incubation period, the medium was removed and replaced with fresh MEM and the cells left incubating for a further 48 h. The medium was replaced daily.

Gene silencing was then analysed using a Western Blotting assay. The Western Blotting assay was used to determine the amount of protein expressed by the treated cells (i.e. ITCH expression) as a measure of gene silencing.

At the end of the gene silencing experiment, cells were harvested via a trypsinization process to detach all of the cells from the flasks. Harvested cells were then lysed by the addition of radioimmunoprecipitation buffer (RIPA , 0.02 mL) and centrifuged at 14,400 rpm for 15 minutes . The supernatant containing the proteins was collected and solubilsed in the Thermo Scientific Halt Protease Inhibitor Cocktail (Thermo Scientific, UK) containing protease inhibitors and phosphatase and EDTA, at a ratio of 1 mL of supernatant to 1 mL of protease inhibitor cocktail. The protein content was then determined using a micro BCA assay kit according to the manufacturers protocol (Thermoscientific, UK).

Once the amount of the protein in each sample of the formulations had been determined, an aliquot of the extracted proteins containing 30μg of protein was analysed via an SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel) electrophoresis experiment run at 200 v for 50 minutes. The gel was then transferred to a nitrocellulose membrane and further blocked with the blocking reagent (5% w/v skimmed milk in PBS – pH = 7.4 - containing 0.1%w/v Tween 20) overnight in the fridge. The membrane was then washed five times over 30 minutes, with PBS containing Tween 20 (0.1 %w/v) – 3 washes and PBS (pH = 7.4) and PBS - 2 washes.

Immunostaining of the membrane then took place, first with the primary antibodies of both anti-Actin (the house keeping gene, 0.2 mg. mL 4 μL) and anti-ITCH (the target gene, 0.25 mg mL^{-1}, 20μL) diluted in Tween 20 (0.1% w/v in PBS, 10 mL). Immunostaining was carried out for 2 hrs at room temperature. The membrane was then rinsed three time with PBS containing Tween 20 (0.1 %w/v) for 5 minutes each time, followed by a further two rinsings with PBS for 5 minutes each time. This was followed by the second labelling with the secondary antibody conjugated with horseradish peroxidase (1 mg mL^{-1}, 10 μL) diluted in Tween 20 (0.1% v/v in PBS, 10 mL). The secondary antibody labelled gel was left to incubate for 1 hr in room temperature. The five washing steps described above were repeated to remove the any residue of the labelling reagents and the blot was developed with SuperSignal west pico chemiluminescent detection kit as per the manufacturer's protocol (Thermoscientific, UK). The membrane was then imaged using a UV camera (Gel Doc XR+ system, Bio-Rad Laboratories, Inc., UK).

In vivo siRNA delivery and gene silencing

For further investigating the delivery of the siRNA with the 4PPA polymer, an in-vivo experiment was carried out. In order to study the behaviour of the nanoparticle complex of the siRNA-4PPA, both qualitatively and quantitatively, a fluorescently labelled siRNA against GAPDH mRNA was chosen as a model. This model siRNA was used to study the delivery of siRNA and the siRNA-ITCH gene chosen for the gene silencing study. The following sections will cover the complex formation, dosing to the animals and the analyses methods for both the localization of the siRNA and the gene silencing experiments.

siRNA Delivery

4PPA with the Cy^3–siRNA-GAPDH complexes were prepared in a similar way to the complex formulations used in the in-vitro experiments in which an aliquot from a stock solution of the 4PPA (40.5 mg mL^{-1}) has been mixed by pipetting with the Cy^3–siRNA-GAPDH solution (1mg mL^{-1}) at a nitrogen to phosphate ratio of 60. This was the best nitrogen to phosphate ratio as identified in the in vitro experiments.

Male Sprague Dawley rats weighing between 220-260g were used for these experiments and these animals were dosed under light anaesthesia using isoflurane (Forthane, Abbot, UK) for 3 – 5 minutes; they were intranasally dosed using a 0.1 mL syringe attached to a flexible 1.5 cm length of tubing. Single rats were either dosed with

formulations containing: a) Cy^3-siRNA-GAPDH alone (1 mg mL^{-1}, 20 μL), b) 4PPA - Cy^3-siRNA-GAPDH (22.5 mg mL^{-1} and 0.444 mg mL^{-1} respectively in 45 μL) or c) 4PPA alone (22.5 mg mL^{-1}, 40 μL). After the animals were dosed, they were placed on their backs (with their heads positioned pointing upwards in order to maximize the residency time of exogenous substances on the olfactory epithelium) and the dose was administered intranasally into one of the nostril over 15-30 seconds. Animals were killed 5 minutes after dosing.

Animals were culled using an overdose of Euthatal® (0.6 mL) followed by cervical dislocation to confirm culling. Brain and Olfactory bulbs were dissected, and snap frozen inside a 50mL falcon tube. All tissues were stored in -80 °C until analyses could be performed.

To visualize the localization of the fluorescent-labelled siRNA, confocal microscopy was utilized for imaging the particles within the brain and olfactory bulb tissues. The olfactory bulbs were left to defrost on the bench. A small smear-like cut of tissue was sampled from the surface of the olfactory bulb. Each sample was handled with a new spatula and the razor wiped in-between samples. For confocal imaging, samples where then placed over a glass slide, fixed with formalin®(4%w/v Formaldehyde) for 10 minutes, followed by a washing cycle and then the application of a mounting anti-fading medium [Vectashield®+DAPI (2-(4-amidinophenyl)-1H -indole-6-carboxamidine – 1.5 mg mL^{-1}), 10-20μL] and the samples were covered with a coverslip gently attached to the mounting medium. Samples were left in fridge overnight in a light-protect box and the following day the slides were imaged using confocal laser scanning microscopy (Carl Zeiss, LSM 710 Confocal Microscopy, Zeiss UK).

In vivo siRNA Gene Silencing

Groups (n = 5) of male Sprague Dawley rats weighing between 220-260g were used for these experiments and these animals dosed under light anaesthesia using isoflurane (Forthane, Abbot, UK) for 3 – 5 minutes; the animals were intranasally dosed using a 0.1 mL syringe attached to a flexible 1.5 cm length of tubing. Groups of rats (n = 5) were dosed twice daily with either formulations containing: a) siRNA-ITCH alone (1 mg mL^{-1}, 40 μL), b) 4PPA - siRNA-ITCH (81 mg mL^{-1} and 1 mg mL^{-1} respectively in 45 μL), c) 4PPA – scrambled siRNA (81 mg mL^{-1} and 1 mg mL^{-1} respectively in 45 μL) or d) PBS (pH = 7.4). After the animals were dosed, they were placed on their backs (with their heads positioned pointing upwards in order to maximize the residency time of

exogenous substances on the olfactory epithelium) and the dose was administered intranasally into one of the nostril over 15-30 seconds. Animals were dosed twice daily for 3 days and then culled 18 hours after the sixth dose was administered. Animals were culled using an overdose of Euthatal® (0.6 mL) followed by cervical dislocation to confirm culling. Brain and Olfactory bulbs were dissected, and snap frozen inside a 50mL falcon tube. All tissues were stored in -80 °C until analyses could be performed.

Brain and olfactory bulb tissue samples were left to defrost, homogenized (VWR® Disposable Pellet Mixers and Cordless Motor) on ice in 20mL of lysis buffer (Tissue Protein Extraction Reagent with Phosphatase and Protease inhibitor – T-PER) for every 1g of tissue. New microtubes were used for each sample to minimize the cross-over between samples. Homogenized tissues were then centrifuged (14,400g, 15 min, 4 °C) to pellet cell and tissues debris and the supernatant collected into new tubes. The protein content was determined using a micro BCA assay kit according to the manufacturers protocol (Thermoscientific, UK).

Once the amount of the protein in each sample of the formulations had been determined, an aliquot of the extracted proteins containing 60µg of protein was analysed via an SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel) electrophoresis experiment run at 200 v for 50 minutes. The gel was then transferred to a nitrocellulose membrane and further blocked with the blocking reagent (5% w/v skimmed milk in PBS – pH = 7.4 - containing 0.1% Tween 20) overnight in the fridge. The membrane was then washed five times over 30 minutes, with PBS (pH = 7.4) and PBS containing Tween 20 (0.1 %w/v) as described above.

Immunostaining of the membrane then took place, first with the primary antibodies of both anti-Actin (the house keeping gene, 0.2 mg mL^{-1}, 4 µL) and anti-ITCH (the target gene, 0.25 mg mL^{-1}, 20µL) diluted in Tween 20 (0.1% w/v in PBS). Immunostaining was carried out for 2 hrs at room temperature. The membrane was then rinsed three time with PBS containing Tween 20 (0.1 %w/v) for 5 minutes each time, followed by a further two rinsings with PBS for 5 minutes each time. This was followed by the second labelling with the secondary antibody conjugated with horseradish peroxidase (1 mg mL^{-1}, 10 µL) diluted in Tween 20 (0.1% v/v in PBS, 10 mL). The secondary antibody labelled gel was left to incubate for 1 hour at room temperature. The five washing steps described above were repeated to remove the any residue of the labelling reagents and the blot was developed with SuperSignal west pico chemiluminescent

detection kit as per the manufacturer's protocol (Thermoscientific, UK). The membrane was then imaged using a UV camera (Gel Doc XR+ system, Bio-Rad Laboratories, Inc., UK).

Results

Experiment A - Polymer Synthesis and Characterisation

Tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-*N*-(2-propylamine)-]-pentaerythritol (4-arm-PEG-propylamine – 4PPA) was prepared in good yield using the chemical synthesis route shown in Figure 8, and then characterised. MALDI-TOF data for the parent 4PEGA (m/z = 2049.3) and for the 4APEA (m/z = 2287) were obtained.

Experiment B - Polyplex Production

4PPA – gene polyplexes were formed over a range of nitrogen to phosphate ratios (Figure 9) as were 4PPA – siRNA complexes, producing nanoparticles in the 200 – 300 nm size range at the higher nitrogen to phosphate ratios (Figures 9 and 10). The complexes presented with a positive zeta potential (Table 3).

Table 3:4PPA – Nucleic Acid Complex Zeta Potentials After 1 hour of Complexation

Formulation	Nitrogen, Phosphate Ratio	Zeta Potential (mV)
4PPA – pDNA containing 25 μg/ mL pDNA	40	18.2
	60	18.4
	80	16.8
	100	17.8
4PPA – siRNA containing 40 μg/ mL siRNA	40	8.6
	60	12.1
	80	13.7

The vector was designed to complex nucleic acids via electrostatic interactions between the cationic charge of the vector and anionic charge of the nucleic acid phosphate groups and this was indeed the case as demonstrated by the production of nanoparticles from a solution of the vector and a solution of the nucleic acid (Figures 9 and 10).

Experiment C – *In vitro* Cytotoxicity

Table 4 – In vitro Cytoxicity against the A431 cell line

Transfection Reagent	IC 50 mg/ mL
4PPA	13.92
Lipofectamine	0.019

4PPA showed good biocompatibility against the A431 cell line and was over 700 times more biocompatible than Lipofectamine, a commercial transfection reagent (Table 4). The polymer was also more than 400 times more biocompatible than the Generation 3 poly(propylenimine) dendrimer transfection reagent, which has an IC50 of 0.03 mg mL $^{-1}$ in the A431 cell line (B.H. Zinselmeyer, S.P. Mackay, A.G. Schatzlein, I.F. Uchegbu, The lower-generation polypropylenimine dendrimers are effective gene-transfer agents, Pharm. Res., 19 (2002) 960-967.) In the MiaPaca2 cell line 4PPA had an IC50 of 9.25 mg mL^{-1} (Table 5). The inventors were able to produce a nucleic acid complexing agent with a favourable IC50.

Table 5 – In vitro cytoxicity against the MiaPaca-2 cell line

Transfection Reagent	IC 50 mg/ mL
4PPA	9.25

A low toxicity index, as measured by the IC50, is desirable in a nucleic acid vector.

Experiment D – *In Vitro* Nucleic Acid Transfer

These 4APPA complexes were able to transfer DNA into cells and as evidenced by the protein expression measured, it may be concluded that these complexes were also able to transfer DNA to the nucleus with a similar efficacy to lipofectamine.

Table 6 – In vitro gene transfer

Formulation	Beta-galactosidase activity (mU per microgram DNA added)
4PPA – pDNA (N, P ratio = 40)	4.79 ± 2.97
4PPA – pDNA (N, P ratio = 60)	5.82 ± 3.52
4PPA – pDNA (N, P ratio = 80)	6.27 ± 4.17
4PPA – pDNA (N, P ratio = 100)	4.43 ± 4.19

Lipofectamine	4.74 ± 3.61
pDNA alone	Below the limit of quantification
Untreated cells	Below the limit of quantification

The 4PPA complexes were also able to transfer siRNA into cells (Figure 11) as shown by the down regulation of the ITCH gene.

The combination of the very good cell biocompatibility and transfection efficacy already proves that DNA vectors may be prepared from molecules with a reduced amine content, such as 4PPA.

Experiment E – *In Vivo* Nucleic Acid Transfer

Figure 12 shows evidence of siRNA within the cells of the olfactory bulb after the intranasal dosing of 4PPA – Cy^3-siRNA-GAPDH. Control animals dosed with Cy^3-siRNA-GAPDH alone do not show high levels of siRNA within the olfactory bulb cells. The inventors have demonstrated that 4PPA is able to deliver siRNA into the olfactory bulb cells of a living rodent on intranasal dosing.

The inventors then went on to examine if the delivered siRNA was pharmacodynamically active using siRNA against the ITCH gene as a model system. Figure 13 shows down regulation in brain tissue of the ITCH gene using 4PPA – siRNA-ITCH. The levels of down regulation were superior to those seen with the controls such as scrambled siRNA and siRNA-ITCH alone. The inventors have thus devised a nucleic acid transfer system that is able to down regulate genes in brain tissue. This was done by using the new nucleic acid gene transfer system and avoiding the blood brain barrier by accessing the brain via the intranasal route.

EXAMPLE 3 – Delivery of mRNA

Synthesis of 4PPA, tetra-O,O,O,O-[poly(ethyleneglycol-O-2-ethyleneimine)-graft-N-(3-propylamine)-]-pentaerythritol

Tetra-O,O,O,O-poly(ethyleneglycol-O-2-ethyleneimine (4APEG, 0.6324 g, 0.316 mmol) was dissolved in Chloroform (37.5 mL). 3-(Boc-amino) propyl bromide (1.26490 g, 5.31 mmol) was dissolved in Chloroform (25 ml) and then added to the 4APEG solution with continuous stirring. Triethylamine (TEA, 6.25 mL, 44.84 mmol) was added to this

mixture and the reaction was refluxed for 51 hours. At the end of this reaction, the solvent was removed by evaporation under reduced pressure at 40 °C.

To the crude product was carefully added HCl (3M, 125 mL) to deprotect the amino groups. The resulting solution was left stirring for 90 mins and eventually dialyzed against water (5L, molecular weight cut off = 1kDa) over a 23 h period and with 5 changes of the dialysis medium. The dialysate was lyophilised and the dried yellow paste was reconstituted in water (6mL).

The resulting solution was neutralized with NaOH powder to an alkaline pH (pH=10). For each 1 mL of sample, 2 mL of CH_2Cl_2 was added and an extraction carried out. This extraction was repeated, the organic phases pooled and dried with anhydrous sodium sulphate. The organic phase was separated evaporated to dryness and the product (4PPA) reconstituted in water and freeze dried. The product was analysed by nuclear magnetic resonance spectrometry and the molecular weight distribution determined by amtrizx assisted laser desorption – time of flight analysis.

mRNA Transfection in the A431 cell line

Cells were seeded in 6 well-plates (300.000 cells per well). The cells were incubated at 37 °C in 5% CO_2 and with Dubelco's Modified Eagle's Medium (DMEM) supplemented with foetal bovine serum (10% FBS).

Phosphate buffer (2mM, pH = 6, PB) was prepared in UltraPure deoxyribonuclease and ribonuclease free water. The 4PPA obtained from above was mixed with beta-galactosidase mRNA (obtained from Trilink Technologies) in the 4PPA, mRNA ratios indicated below and the final concentration of mRNA was 1 µg/mL. The mRNA solution was mixed with the polymer solutions to obtain 4PPA-mRNA formulations in the following 4PPA, mRNA weight ratios:

4PPA-mRNA (7: 1) mRNA → 1.5 µL (1mg/ml) + 748.5 µL PB
4PPA → 20 µL (0.5mg/ml) + 730 µL PB

4PPA-mRNA (16: 1) mRNA→ 1.5 µL (1mg/ml) + 748.5 µL PB
4PPA→ 48.8 µL (0.5mg/ml) + 701.2 µL PB

4PPA-mRNA (26: 1) mRNA→ 1.5 µL (1mg/ml) + 748.5 µL PB

4PPA→ 80 µL (0.5mg/ml) + 670 µL PB

The medium was removed and the cells washed twice with phosphate buffered saline (PBS) and to each well was added medium (1.5 mL, without serum) and the mRNA formulation (0.5 mL). The well containing cells alone was incubated with 2 mL of medium without serum. After 4 h the formulations were removed and the medium plus serum added. The cells were incubated for 24 h and the protein then extracted and measured according to the manufacturer's protocol (Promega, UK).

Table 7 - Transfection results

Measure	4PPA – mRNA (7: 1)	4PPA – mRNA (16: 1)	4PPA – mRNA (26: 1)	Cells alone	mRNA alone
mU β-galactosidase per well	0.01	0.08	0.01	below the limit of quantification	below the limit of quantification

Discussion

The described work was aimed at improving the success of gene therapy as to date only two approved products exist, despite the gene therapy first being demonstrated as a concept almost a quarter of a century ago and the first report of a gene therapy cure in humans being reported almost 15 years ago. The approved gene therapy products are delivered using viruses and are injected locally. There is thus still a knowledge gap surrounding the safe systemic delivery of gene payloads in humans, particularly if synthetic vectors are used. There has been recent encouragement however with the systemically administered siRNA therapy, which is in clinical trials, however no commercial products yet exist. Clinical and preclinical studies have not yet yielded synthetic gene therapy formulations that have then progressed to registration due to a combination of poor efficacy and the questionable safety of such agents. The inventors have produced a new synthetic gene vector, which was specifically designed to be more biocompatible than previous vectors and yet maintain gene transfer activity.

4APEA and 4PPA are synthetic vectors designed with the knowledge gained from the development of the Generation 3 poly(propylenimine) dendrimers (molecular weight = 1687 Da) as gene therapy vectors (Dufes, C. et al. *Cancer Res.* 2005, 65, (18), 8079-8084; Chisholm, E. J. et al *Cancer Res.* 2009, 69, 2955-2962). 4APEA and 4PPA are thus relatively low molecular weight (molecular weight ~ 2000 Da) branched polymers

which are biocompatible because they contain fewer amine groups per molecule (Figures 2 and 8) when compared to the PPI dendrimers. The inventors have demonstrated that a reduction in amine content by starting from a poly(ethylene glycol) molecule, which was subsequently decorated with amine groups, produces a more biocompatible molecule with cell IC50 values of ~ 4 – 13 mg mL^{-1} (Tables 2 and 5). Surprisingly, 4APEA and 4PPA are over 1000 times less cytotoxic than the gene transfer agent – PEI (Brownlie, A. et al., *Int. J. Pharm.* **2004,** 274, (1-2), 41-52) and over 400 times less toxic than the Generation 3 poly(propylenimine) dendrimer (Zinselmeyer, B. H. et al. *Pharm. Res.* **2002,** 19, (7), 960-967).

Amines, which are positively charged at neutral pH, are required for electrostatic complexation with DNA as DNA has a negative charge at neutral pH. Moving from 4 to about 8 amine units per molecule enables 4APEA to complex DNA and form 200 nm positively charged nanoparticles (Figure 3a, 3b, 3c). 4APEA and 4PPA forms 100 - 300 nm nanoparticles (Figures 3 and 10). These particles transfer DNA into the nucleus and give rise to the expression of exogenous proteins (Figure 5 and Table 6). One notable feature of the system is that, due to the low amine content in each individual molecule, to achieve a transfection competent nanoparticle with the required nitrogen to phosphate ratio of 40 : 1 requires a polymer to DNA weight ratio of 28: 1, which compares unfavourably to the Generation 3 PPI dendrimer, in which an N, P ratio of 30: 1 is achieved with a dendrimer, DNA weight ratio of 5: 1 (Dufes, C. et al, *Cancer Res.* **2005,** 65, (18), 8079-8084). However, the biocompatibility gains that may be achieved with these new polymers are considerable and hence *in vivo* testing was considered warranted.

The inventors tested their model system 4PPA in a pancreatic cancer tumour model in order to capitalise on the fact that gene transfer to the nucleus occurs when cells are dividing, a fact that could improve the therapeutic index of a formulation designed to generate the relatively toxic TNF-alpha protein in cancer therapies. It is generally accepted that a longer blood residence time favours distribution to tumour tissue, as is seen with the liposome technologies both in preclinical and clinical studies, hence the rapid clearance of the particles from the blood will impact negatively on tumour distribution as is seen here (Figure 6). The complexes were rapidly distributed to the lung (Figure 6), as has been seen with other polyamine transfer systems, such as PEI as well as with cationic liposomes, where transfection occurs predominantly in the lung with both classes of agents. This preferential lung transfection with polyamine vectors

is hypothesised to be due to particle aggregation within the vasculature and the eventual trapping of the particles in the lung capillaries, presumably following the coating of the positively charged complexes with plasma and serum proteins (Table 1). Although aggregation is not seen *in vitro* on incubation of serum proteins with 4APEA – DNA complexes, the comparatively higher protein, complex ratio *in vivo* could have given rise to a certain degree of aggregation *in vivo* and the eventual trapping of 4APEA – TNF-alpha gene complexes in the lung capillaries. The complexes also distribute to the liver, the next capillary bed encountered on intravenous injection. Liver transfection has been seen predominantly with the Generation 3 PPI dendrimers.

Importantly, there was also tumour uptake of the complexes. However, with reference to the *in vitro* data (Figure 5), DNA alone would be expected to transfect cells poorly *in vivo*, whereas any of the complex distributed to the tumour tissues has a good chance of transfecting cells efficiently (Figure 5). This is borne out by the data as the activity of the tumouricidal gene is seen in the xenograft model (Figure 7) with only the 4APEA – TNF-alpha complexes showing tumour growth suppression that was significantly different from the untreated controls. The activity of the TNF-alpha gene alone is not significantly different from the untreated controls. The inventors have therefore shown that a relatively biocompatible gene transfer systems may be prepared using polymers based on PEG amine molecules.

The inventors tested their model system, 4PPA, as a neuronal tissue gene silencing agents (Figure 13) and observed gene silencing in brain tissue when the formulations were administered via the intranasal route, making the systems useful for the treatment of neurological diseases and brain cancers.

Conclusions

The 2 kDa star shaped poly(ethylene glycol) (PEG) based amines of the invention containing at least 8 amine groups per molecule, show good biocompatibility with IC50 values in excess of 4 mg mL^{-1} and are able to condense DNA and protect it from serum degradation. However, PEG amines containing only 4 amine groups per molecule do not condense DNA and protect it from serum degradation. Both of the above classes of low molecular weight PEG amines transfer DNA into cells. PEG amine complexes divert DNA to the liver and lung on intravenous administration. Tumouricidal gene PEG amine complexes are tumouricidal in a mouse pancreatic cancer xenograft models.

The 2kDa star shaped poly(ethylene glycol) (PEG) based amines of the invention derivatised with propylamine were able to transfer siRNA to brain tissue on intranasal dosing and bring about gene silencing.

In summary, therefore, the use of the low molecular weight PEG based amine of the invention results in an extremely biocompatible gene transfer agent with both *in vitro* and *in vivo* activity. The PEG based amine of the invention causes a suppression of tumour growth when complexed with a tumouricidal gene or gene silencing in the brain when complexed with the relevant siRNA.

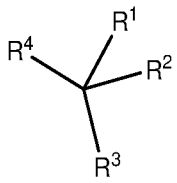
Claims

1. A compound comprising poly(ethylene glycol) amine having a molecular weight of less than 5000 Daltons, wherein the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:44.

2. A compound according to claim 1, wherein the compound has a molecular weight of less than 4000 Daltons, 3000 Daltons, or 2500 Daltons.

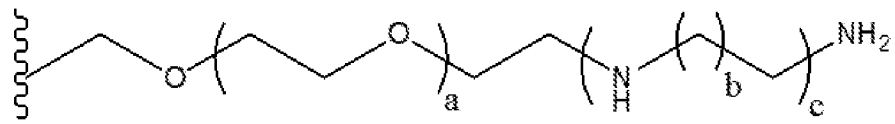
3. A compound according to either claim 1 or claim 2, wherein the ratio of ethylene glycol units to nitrogen atoms is between 30:3 and 30:40, between 30:3 and 30:35, between 30:3 and 30:30, between 30:3 and 30:25, between 30:3 and 30:20, between 30:3 and 30:15, or between 30:3 and 30:10.

4. A compound according to any preceding claim, wherein the compound has Formula (I):



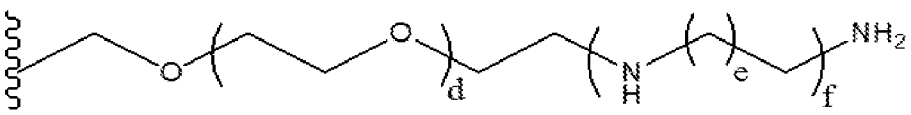
Formula (I)

wherein R^1 is hydrogen or:



wherein a is an integer between 1 and 42,
b is 1 or 2, and
c is an integer between 0 and 10;

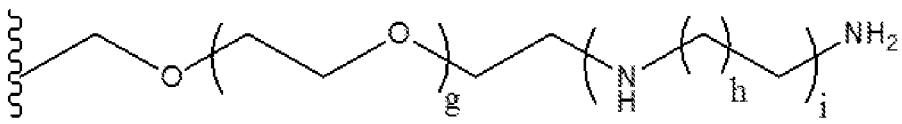
R^2 is hydrogen or:



wherein d is an integer between 1 and 42,

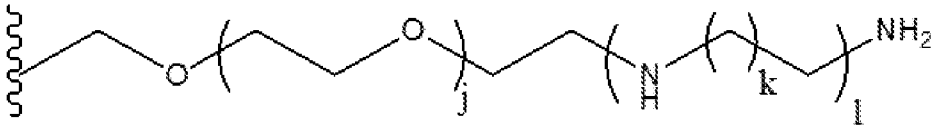
e is 1 or 2, and
f is an integer between 0 and 10;

R^3 is hydrogen or:



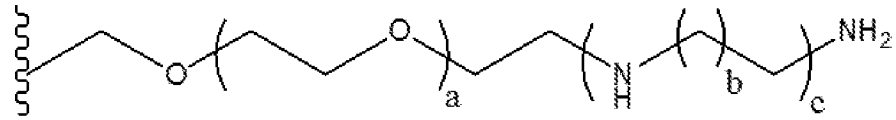
wherein g is an integer between 1 and 42,
h is 1 or 2, and
i is an integer between 0 and 10; and

R^4 is hydrogen or:



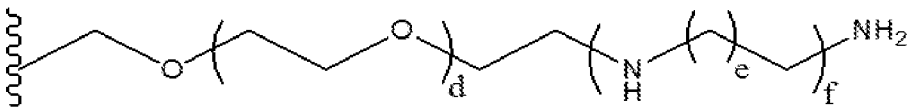
wherein j is an integer between 1 and 42,
k is 1 or 2, and
l is an integer between 0 and 10;
wherein the sum of a, d, g and j is between 30 and 50 and the sum of c, f, i and l is between 1 and 40.

5. A compound according to claim 4, wherein R^1 is:



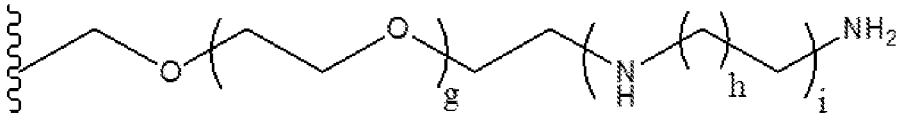
and a is an integer between 2 and 30, between 3 and 25, between 4 and 20, or between 5 and 15;

R^2 is:



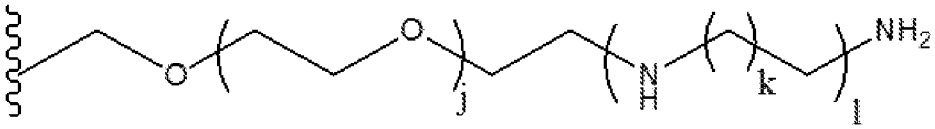
and d is an integer between 2 and 30, between 3 and 25, between 4 and 20, or between 5 and 15;

R^3 is:



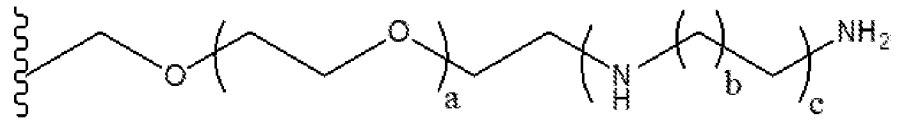
and g is an integer between 2 and 30, between 3 and 25, between 4 and 20, or between 5 and 15; and

R^4 is:



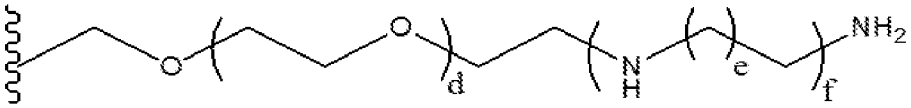
and j is an integer between 2 and 30, between 3 and 25 between 4 and 20, or between 5 and 15.

6. A compound according to either claim 4 or claim 5, wherein R^1 is:



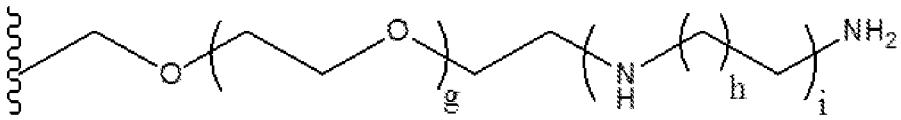
and c is an integer between 0 and 5;

R^2 is:



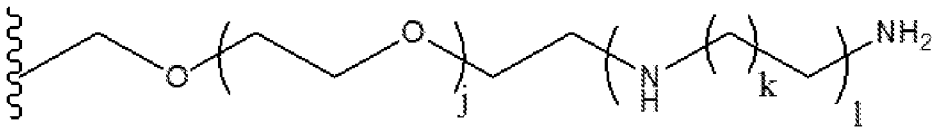
and f is an integer between 0 and 5;

R^3 is:



and i is an integer between 0 and 5; and

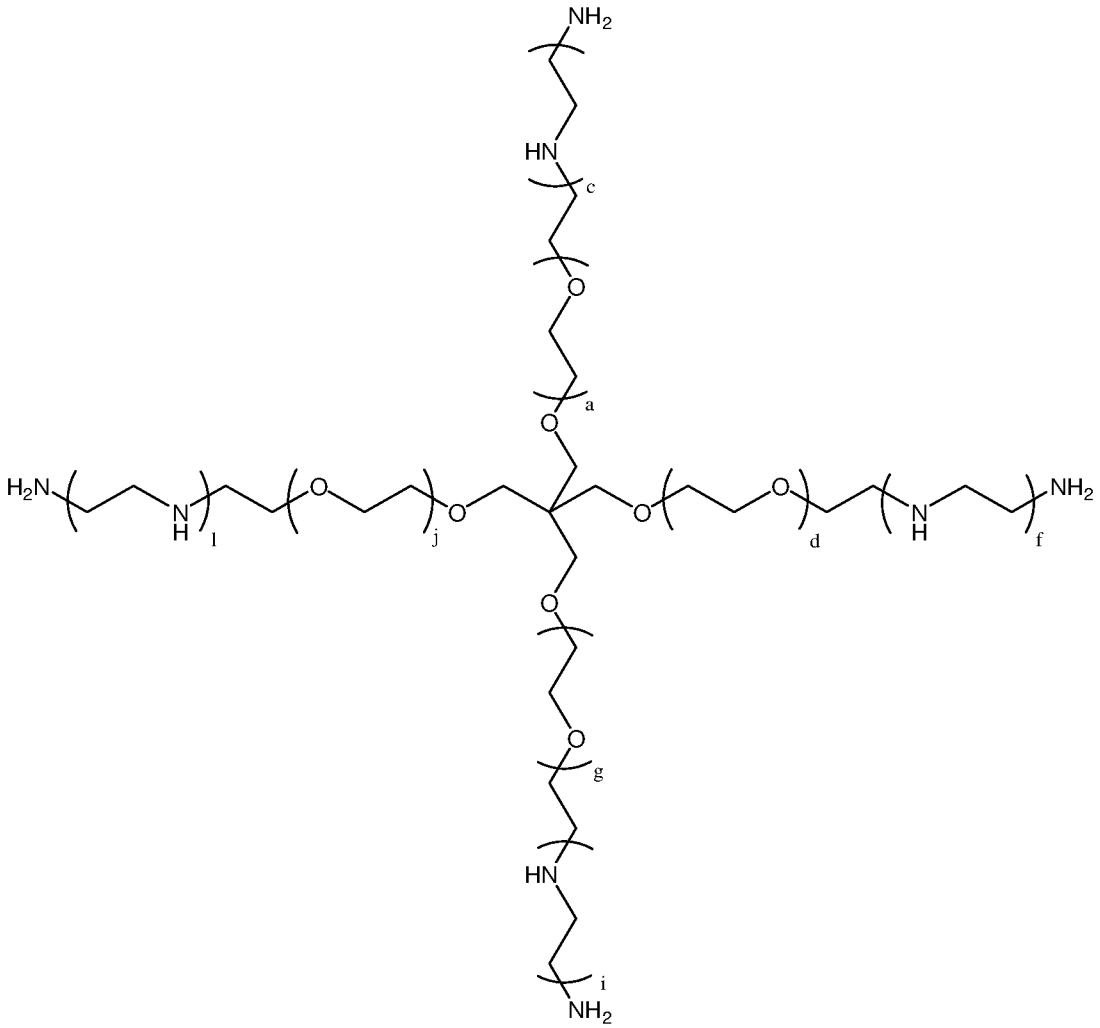
R^4 is:



and l is an integer between 0 and 5.

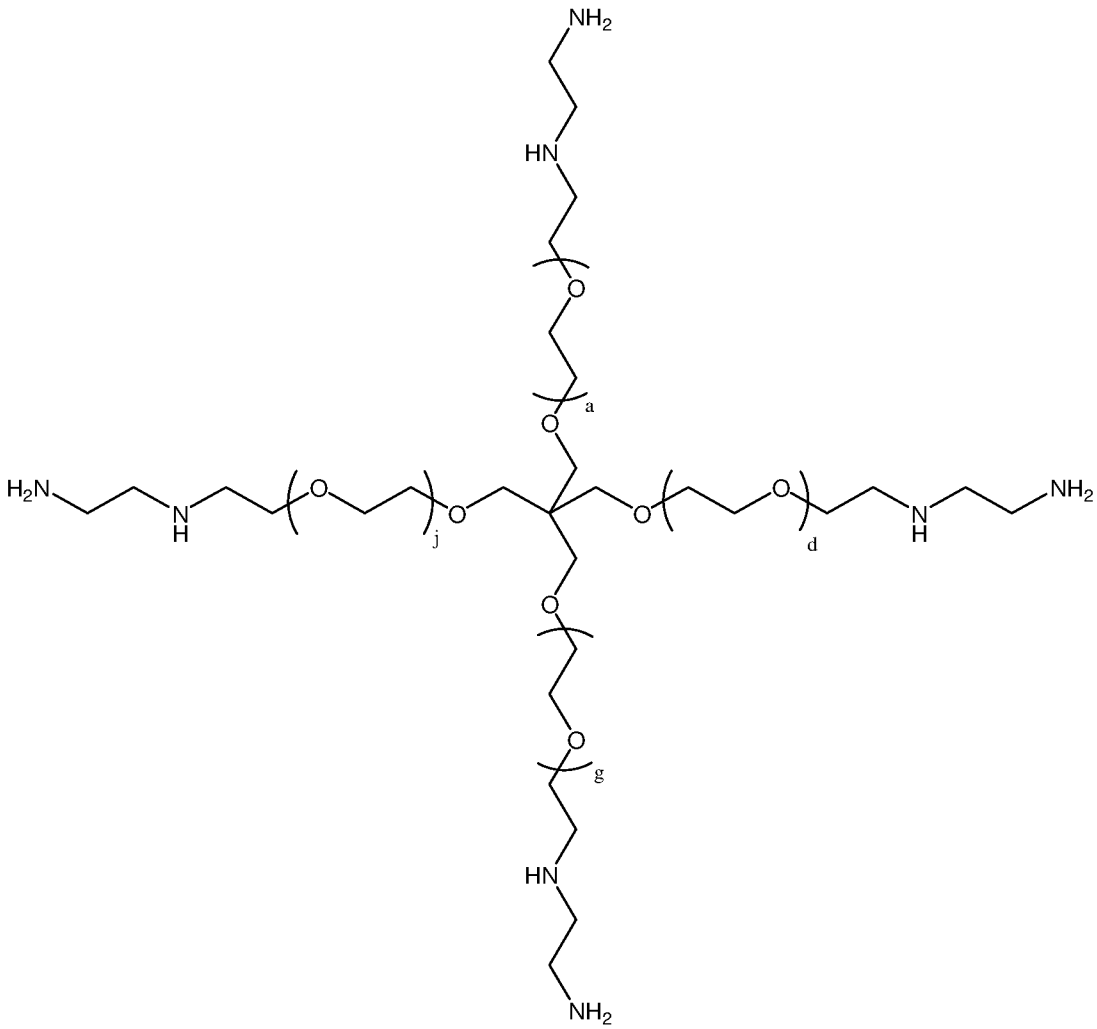
7. A compound according to any one of claims 4 to 6, wherein the sum of c, f, i and l is between 1 and 30, 2 and 20, or 4 and 10.

8. A compound according to any one of claims 4 to 7, wherein b is 1, e is 1, h is 1 and k is 1, and the compound has Formula (Ia):



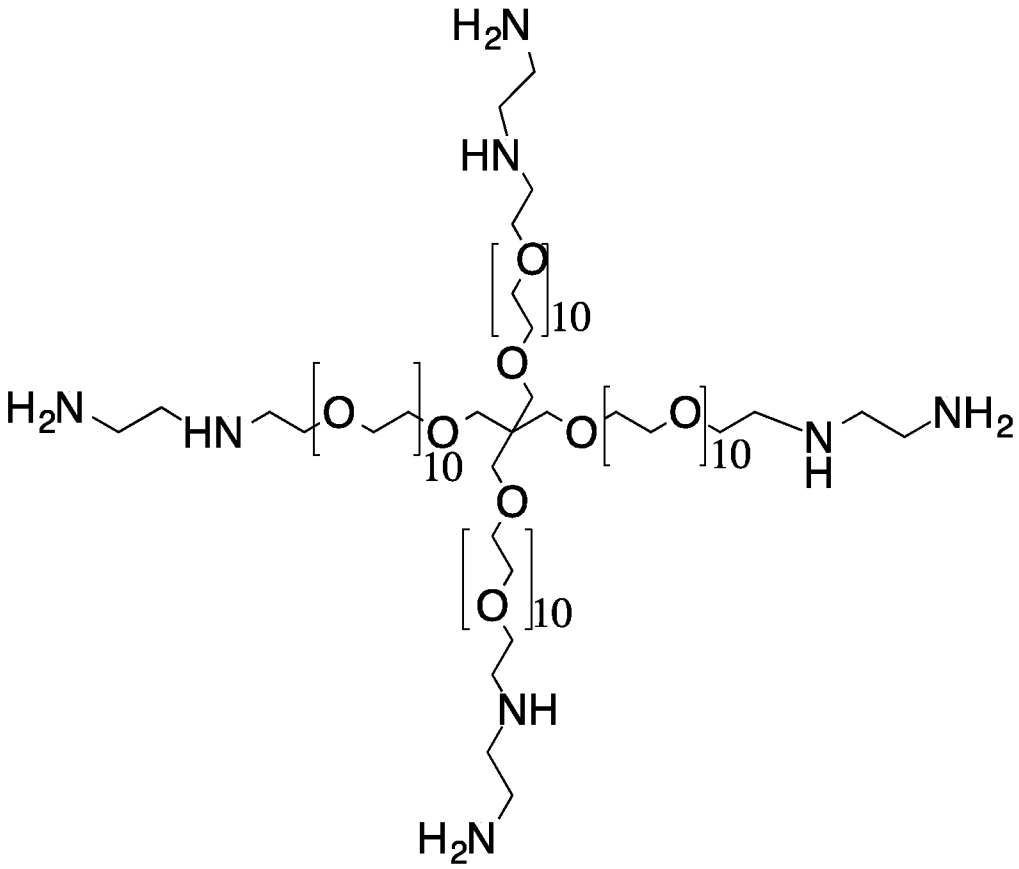
Formula (Ia).

9. A compound according to claim 8, wherein c is 1, f is 1, i is 1 and l is 1, and the compound has Formula (Ib):



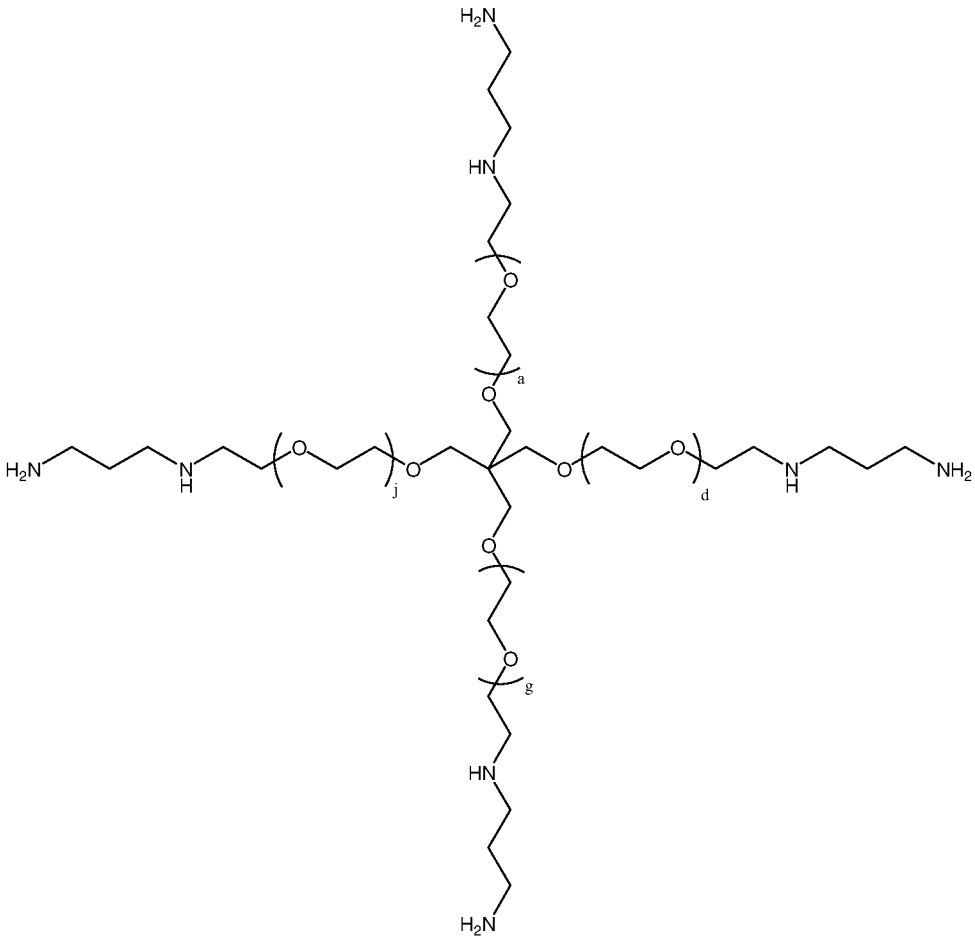
Formula (Ib).

10. A compound according to claim 9, wherein the compound has Formula (Ic):



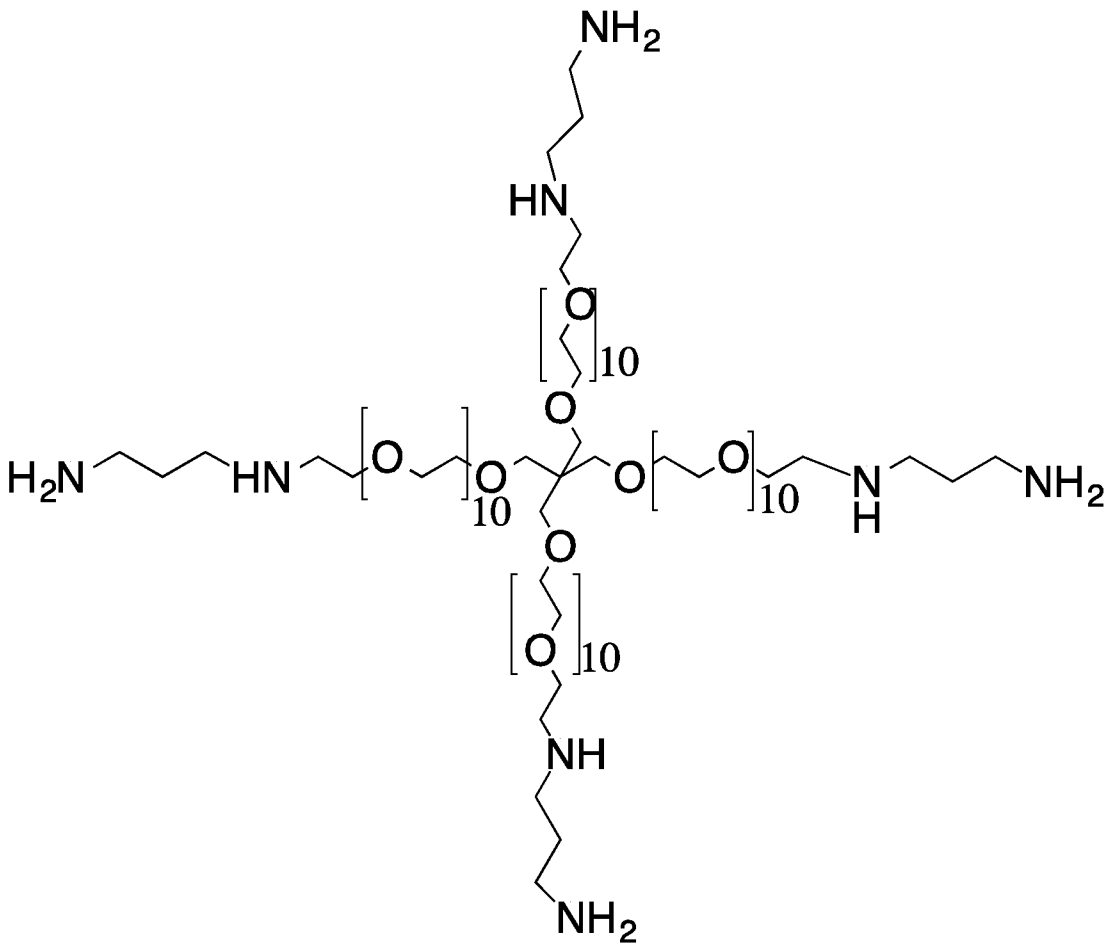
Formula (Ic).

11. A compound according to any one of claims 4 to 7, wherein b is 2, e is 2, h is 2, k is 2, c is 1, f is 1, i is 1 and l is 1, and the compound has Formula (Id):



Formula (Id).

12. A compound according to claim 11, wherein the compound has Formula (Ie):



Formula (Ie).

13. Use of the compound of any one of claims 1 to 12, as a nucleic acid delivery system.

14. A method of producing a nucleic acid delivery complex, the method comprising contacting the compound of any of claims 1 to 12 with a nucleic acid to thereby form a stable nucleic acid delivery complex.

15. A method according to claim 14, wherein the PEG amine - nucleic acid complex is prepared by mixing the nucleic acid and PEG amine in a suitable buffer and allowing the mixture to incubate at about room temperature for at least 10 minutes, 15 minutes or 1 hour.

16. A nucleic acid delivery complex comprising a compound according to any of claims 1 to 12 and a nucleic acid.

17. A nucleic acid delivery complex according to claim 16, wherein the nucleic acid is DNA.

18. A nucleic acid delivery complex according to claim 16, wherein the nucleic acid is RNA.

19. A nucleic acid delivery complex according to any one of claims 16 to 18, wherein the zeta-potential of the complex is at least + 5 mV at neutral pH and does not exceed + 90 mV at neutral pH.

20. A nucleic acid delivery complex according to any one of claims 16 to 19, wherein complex is in the form of a nanoparticle, with an average diameter of more than about 100nm and less than about 900nm.

21. A compound according to any one of claims 1 to 12, or a complex according to any one of claims 16 to 20, for use in therapy or diagnosis.

22. A compound according to any one of claims 1 to 12, or a complex according to any one of claims 16 to 20, for use in a gene therapy technique.

23. A pharmaceutical composition comprising the compound of any one of claims 1 to 12 or the complex of any one of claims 16 to 20, and a pharmaceutically acceptable vehicle.

24. A process for making the pharmaceutical composition of claim 23, the process comprising contacting a therapeutically effective amount of the compound of any one of claims 1 to 12 or the complex of any one of claims 16 to 20, and a pharmaceutically acceptable vehicle.

Figure 1

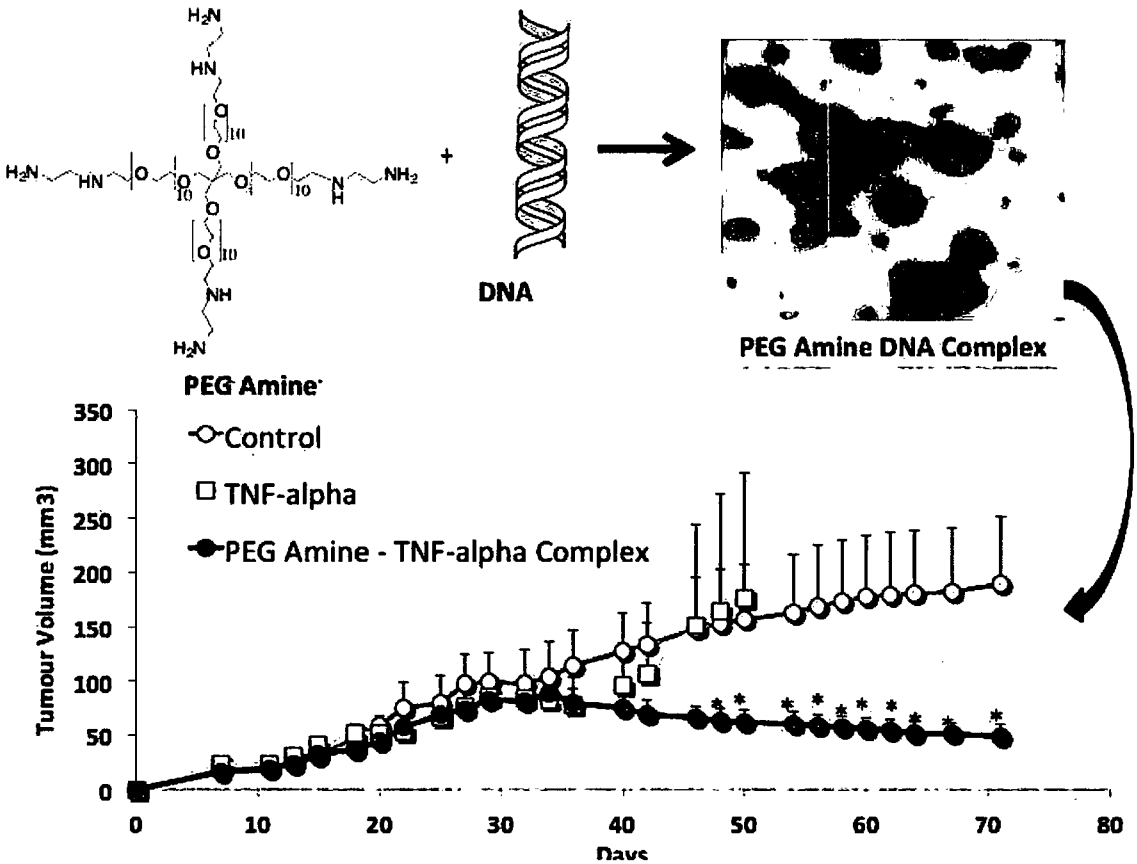

PEG Amine
DNA
PEG Amine DNA Complex
Control
TNF-alpha
PEG Amine - TNF-alpha Complex
Tumour Volume (mm3)
Days
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
70
80

Figure 2

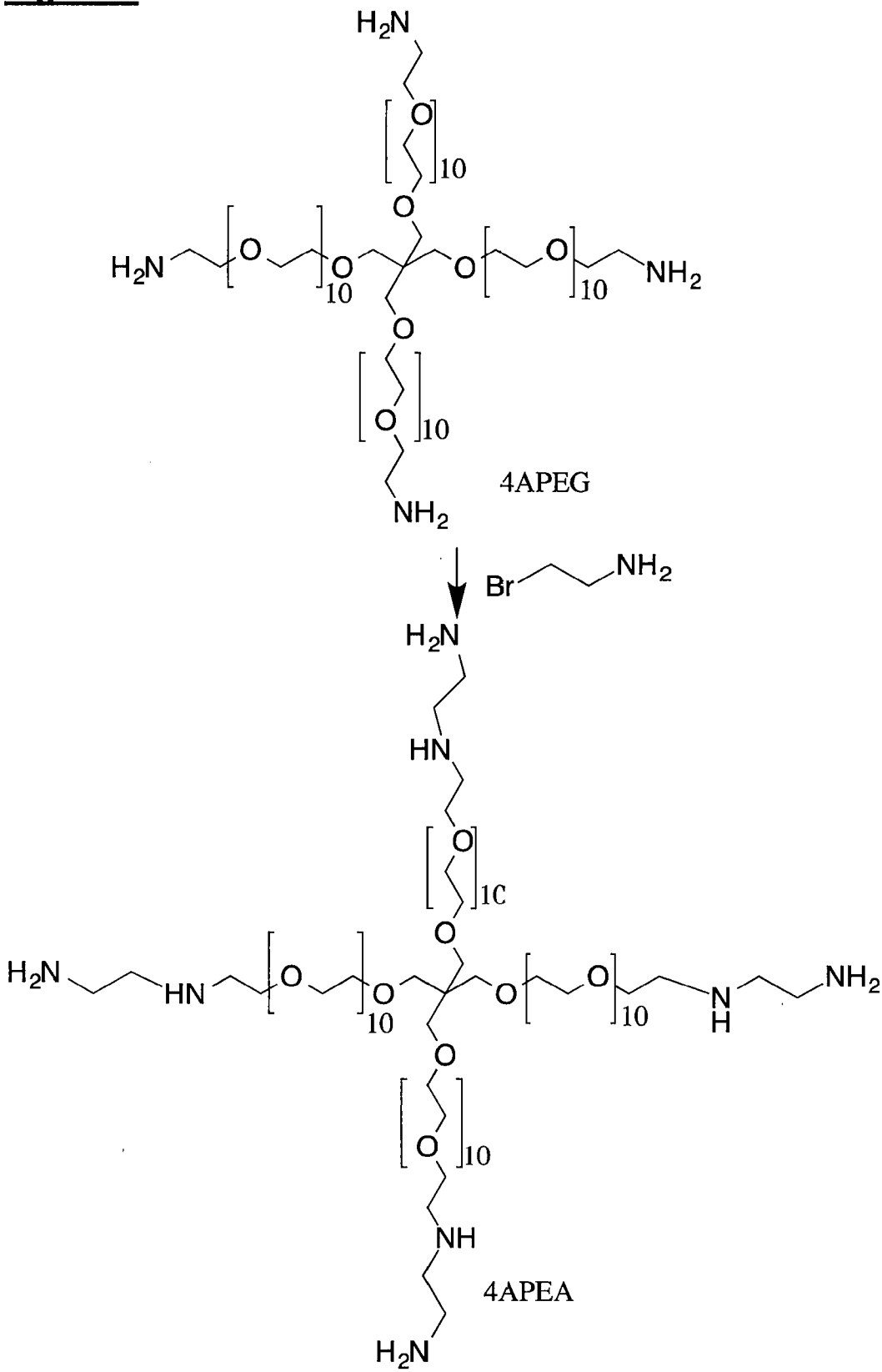

H2N
O
10
O
H2N
O
10
O
O
10
NH2
O
O
10
NH2
4APEG
Br
NH2
H2N
HN
O
10
O
H2N
HN
O
10
O
O
10
N
H
NH2
O
O
10
NH
4APEA
H2N

Figure 3a

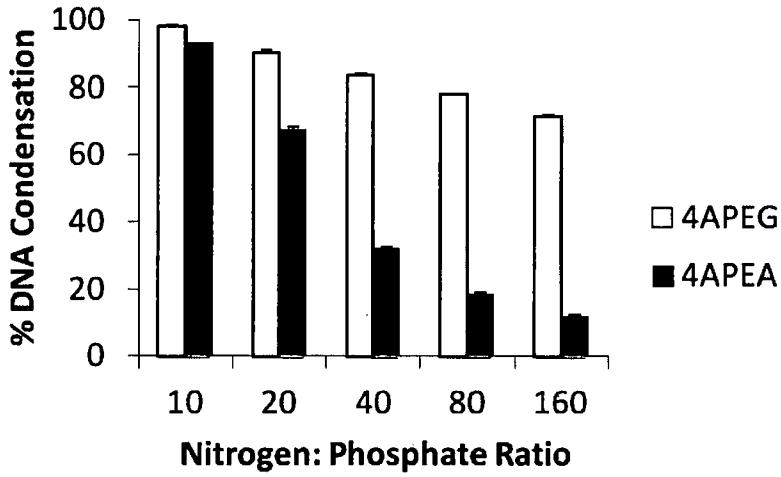


Figure 3b

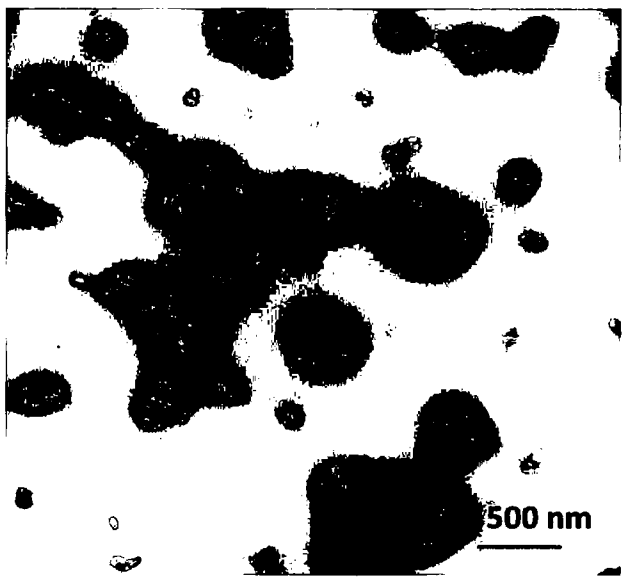


Figure 3c

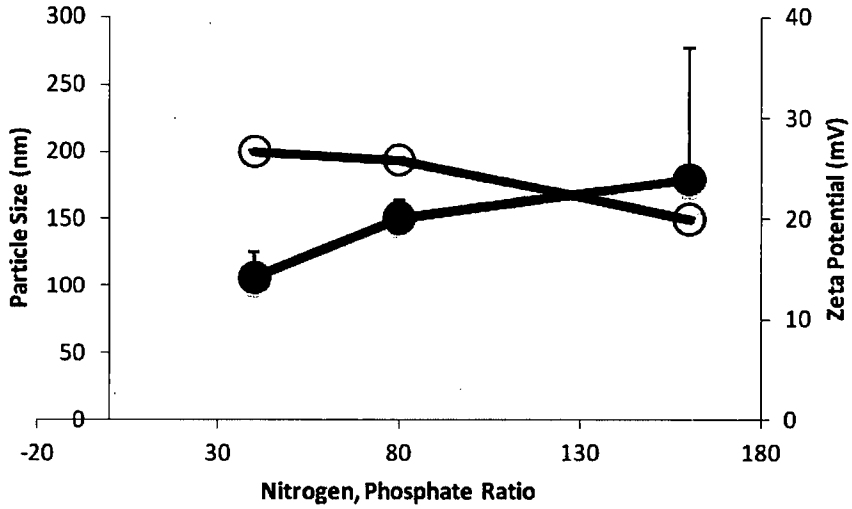
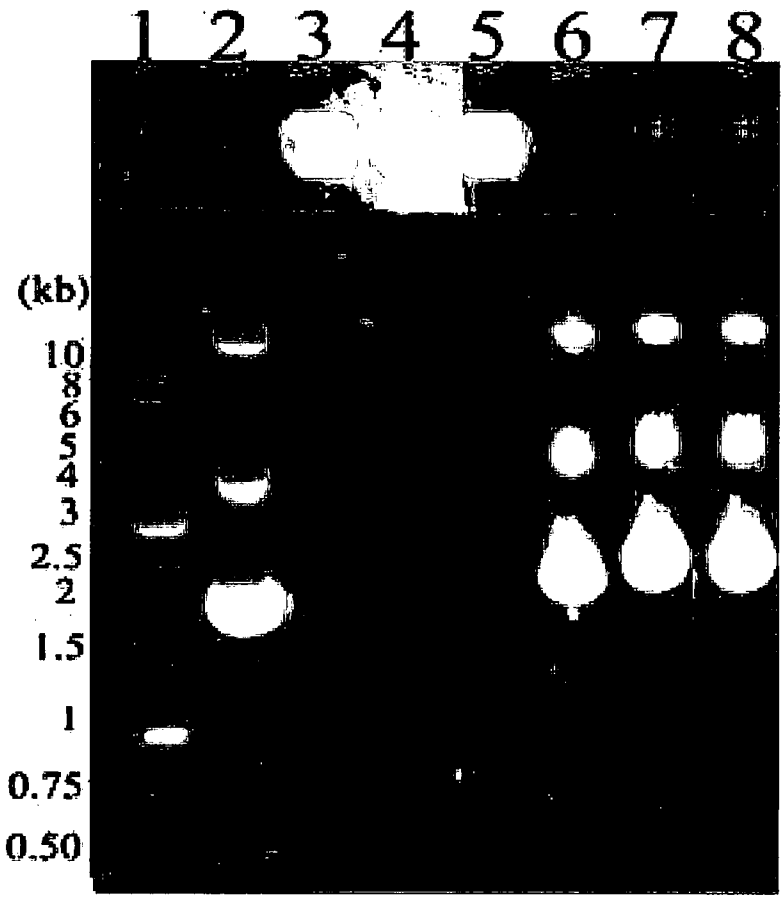
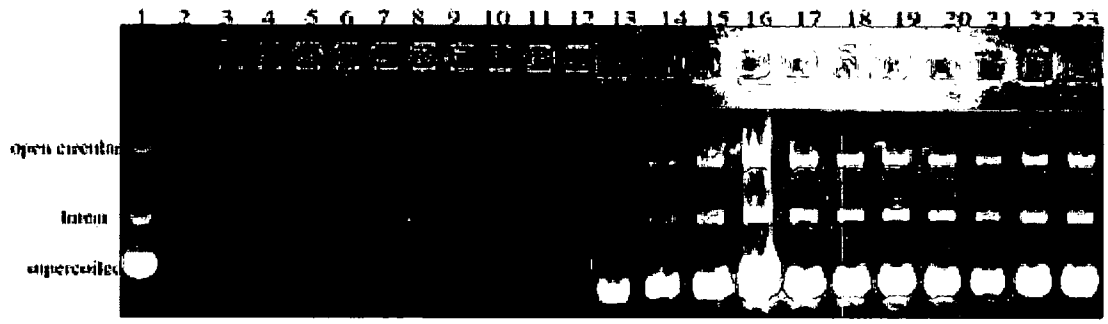

300
250
200
150
100
50
0
Particle Size (nm)
40
30
20
10
0
Zeta Potential (mV)
-20
30
80
130
180
Nitrogen, Phosphate Ratio

Figure 4a



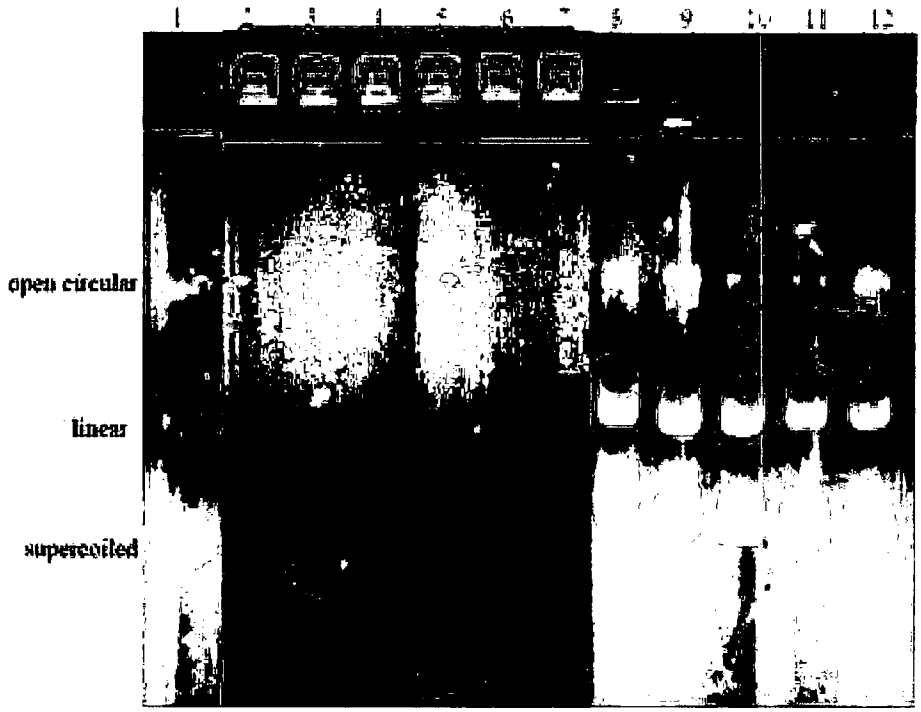
Lane 1: DNA ladder
Lane 2: Naked DNA
Lane 3: 4APEA – DNA (N, P ratio = 40)
Lane 4: 4APEA – DNA (N, P ratio = 80)
Lane 5: 4APEA – DNA (N, P ratio = 160)
Lane 6: 4APEG – DNA (N, P ratio = 40)
Lane 7: 4APEG – DNA (N, P ratio = 80)
Lane 8: 4APEG – DNA (N, P ratio = 160)

Figure 4b



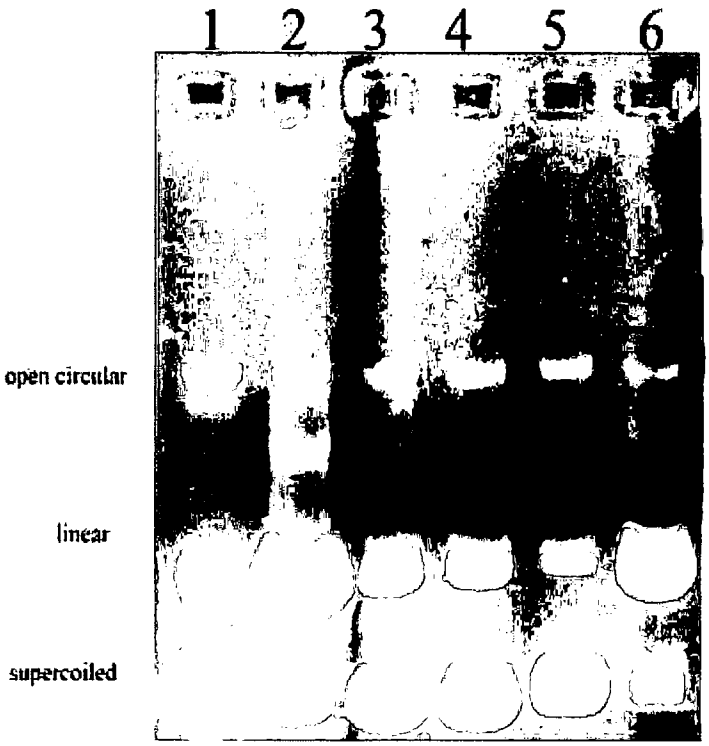
Lane 1: Naked DNA
Lane 2: 4APEA – DNA (N, P ratio = 80)
Lane 3: 4APEA - DNA (N, P ratio = 80) + heparin (0.1 mg mL^{-1})
Lane 4: 4APEA - DNA (N, P ratio = 80) + heparin (0.2 mg mL^{-1})
Lane 5: 4APEA - DNA (N, P ratio = 80) + heparin (0.3 mg mL^{-1})
Lane 6: 4APEA - DNA (N, P ratio = 80) + heparin (0.4 mg mL^{-1})
Lane 7: 4APEA - DNA (N, P ratio = 80) + heparin (0.5 mg mL^{-1})
Lane 8: 4APEA - DNA (N, P ratio = 80) + heparin (0.6 mg mL^{-1})
Lane 9: 4APEA - DNA (N, P ratio = 80) + heparin (0.7 mg mL^{-1})
Lane 10: 4APEA - DNA (N, P ratio = 80) + heparin (0.8 mg mL^{-1})
Lane 11: 4APEA - DNA (N, P ratio = 80) + heparin (0.9 mg mL^{-1})
Lane 12: 4APEA - DNA (N, P ratio = 80) + heparin (1.0 mg mL^{-1})
Lane 13: 4APEG – DNA (N, P ratio = 80)
Lane 14: 4APEG – DNA (N, P ratio = 80) + heparin (0.1 mg mL^{-1})
Lane 15: 4APEG – DNA (N, P ratio = 80) + heparin (0.2 mg mL^{-1})
Lane 16: 4APEG – DNA (N, P ratio = 80) + heparin (0.3 mg mL^{-1})
Lane 17: 4APEG – DNA (N, P ratio = 80) + heparin (0.4 mg mL^{-1})
Lane 18: 4APEG – DNA (N, P ratio = 80) + heparin (0.5 mg mL^{-1})
Lane 19: 4APEG – DNA (N, P ratio = 80) + heparin (0.6 mg mL^{-1})

Figure 4c



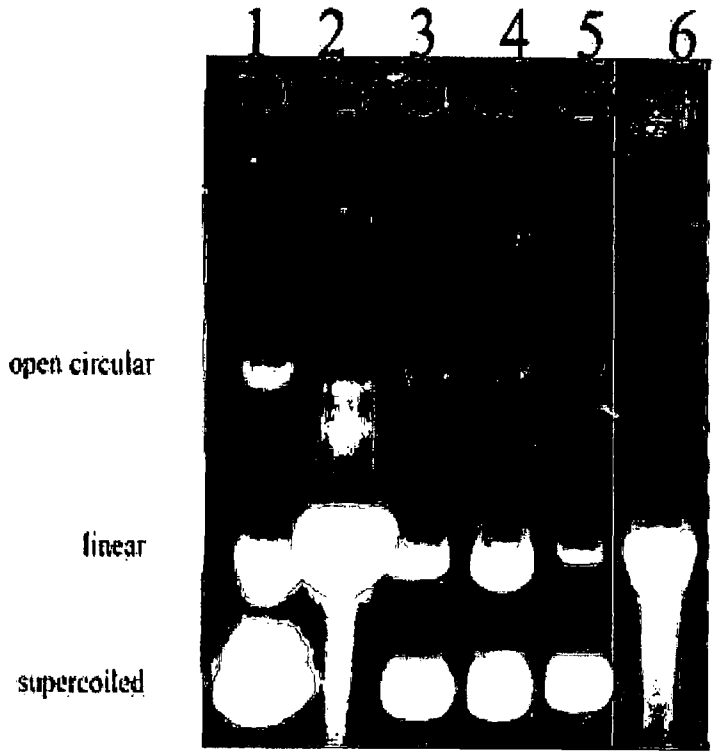
Lane 1: Naked DNA
Lane 2: 4APEA – DNA (N, P ratio = 80)
Lane 3: 4APEA - DNA (N, P ratio = 80) + heparin (0.1 mg mL^{-1})
Lane 4: 4APEA - DNA (N, P ratio = 80) + heparin (0.25 mg mL^{-1})
Lane 5: 4APEA - DNA (N, P ratio = 80) + heparin (0.4 mg mL^{-1})
Lane 6: 4APEA - DNA (N, P ratio = 80) + heparin (0.75 mg mL^{-1})
Lane 7: 4APEA - DNA (N, P ratio = 80) + heparin (1.0 mg mL^{-1})
Lane 8: 4APEA - DNA (N, P ratio = 80) + heparin (2.0 mg mL^{-1})
Lane 9: 4APEA - DNA (N, P ratio = 80) + heparin (2.5 mg mL^{-1})
Lane 10: 4APEA - DNA (N, P ratio = 80) + heparin (3.0 mg mL^{-1})
Lane 11: 4APEA - DNA (N, P ratio = 80) + heparin (4.0 mg mL^{-1})
Lane 12: 4APEA - DNA (N, P ratio = 80) + heparin (5.0 mg mL^{-1})

Figure 4d



Lane 1: Naked DNA
Lane 2: Naked DNA + serum after 1 h.
Lane 3: 4APEA - DNA (N, P ratio = 80) + heparin (5mg mL^{-1})
Lane 4: 4APEA – DNA (N, P ratio = 80) + serum after 1h
Lane 5: 4APEG – DNA (N, P ratio = 80) + heparin (5mg mL^{-1})
Lane 6: 4APEG – DNA (N, P ratio = 80) + serum after 1h

Figure 4e



Lane 1: Naked DNA
Lane 2: Naked DNA + serum after 2 h
Lane 3: 4APEA - DNA (N, P ratio = 80) + heparin (5mg mL^{-1})
Lane 4: 4APEA – DNA (N, P ratio = 80) + serum after 2h
Lane 5: 4APEG – DNA (N, P ratio = 80) + heparin (5mg mL^{-1})
Lane 6: 4APEG – DNA (N, P ratio = 80) + serum after 2h

Figure 5

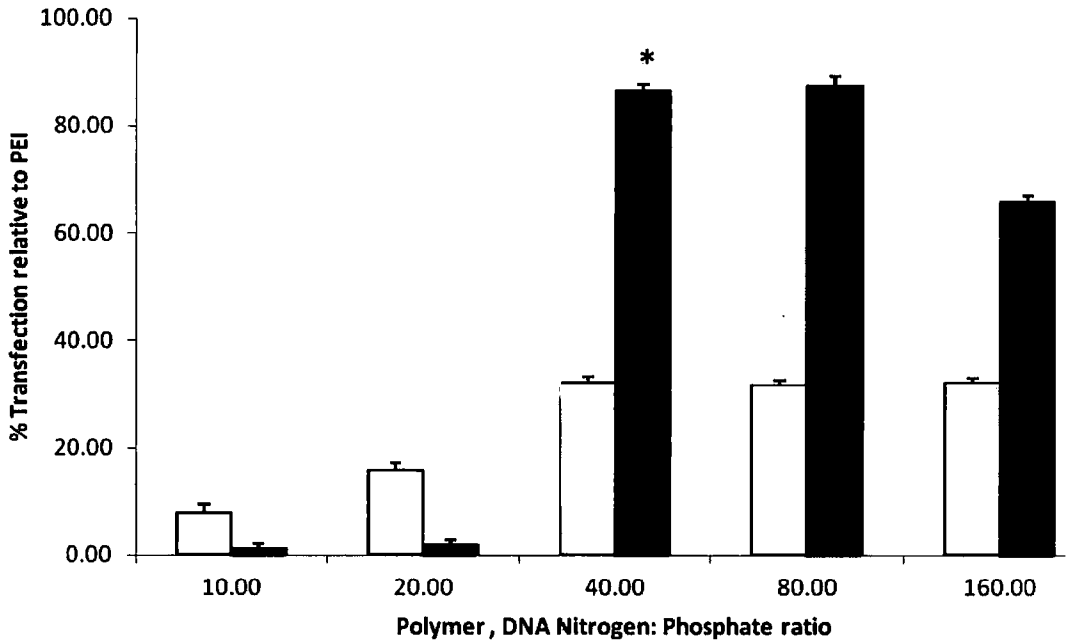


Figure 6a

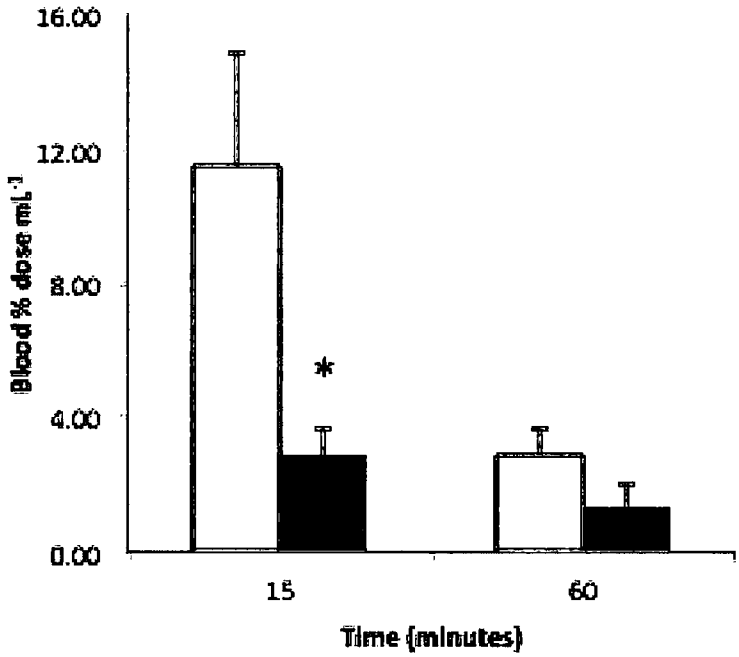


Figure 6b

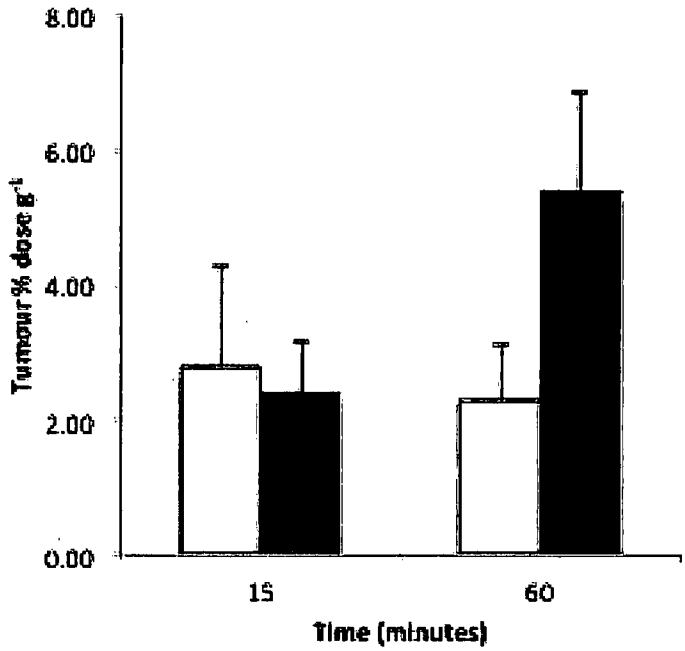


Figure 6c

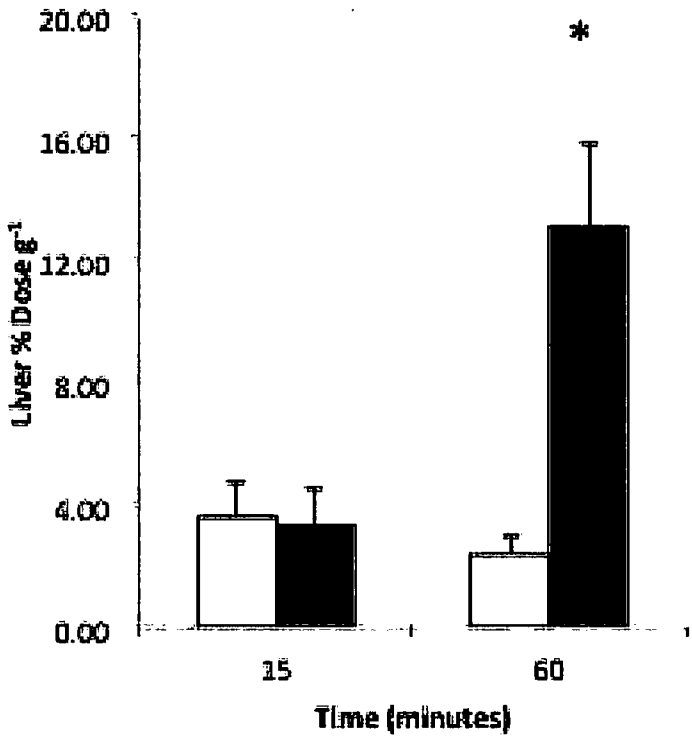


Figure 6d

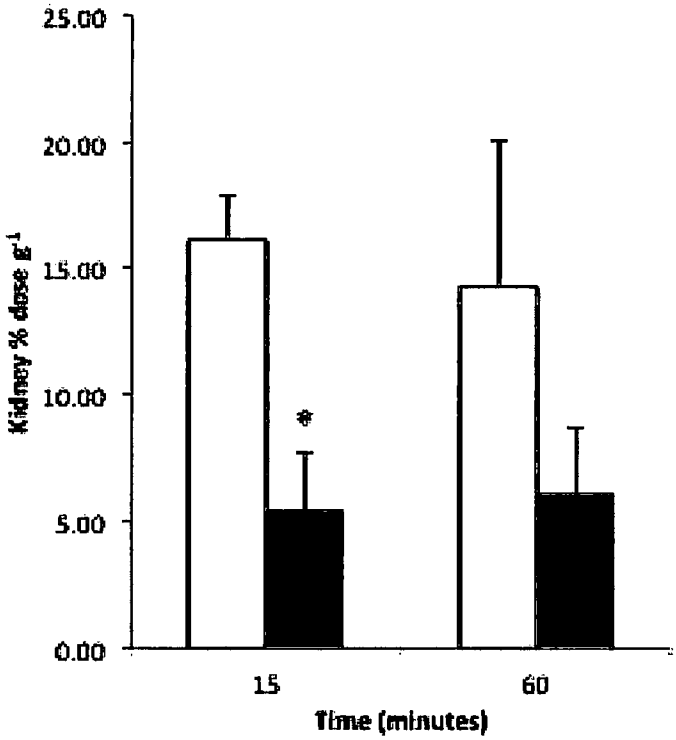


Figure 6e

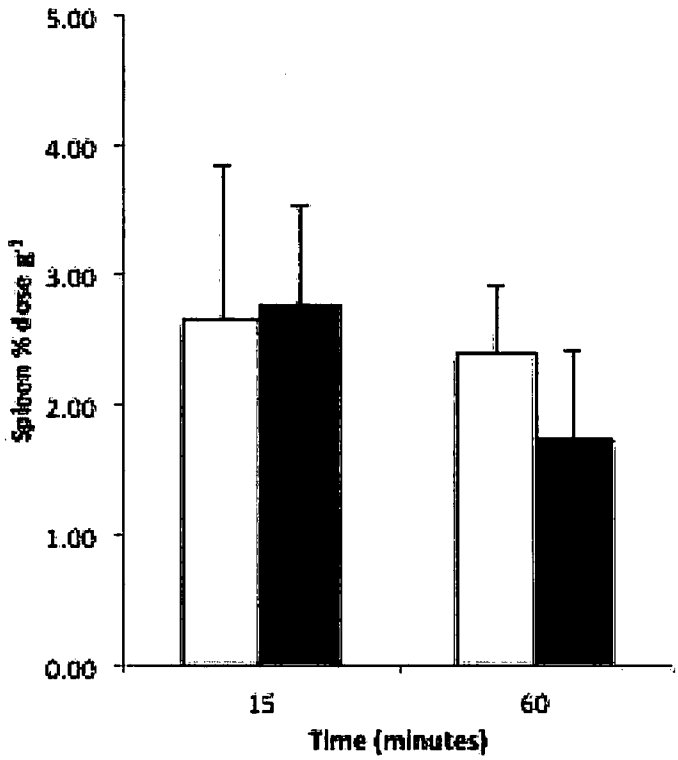


Figure 6f

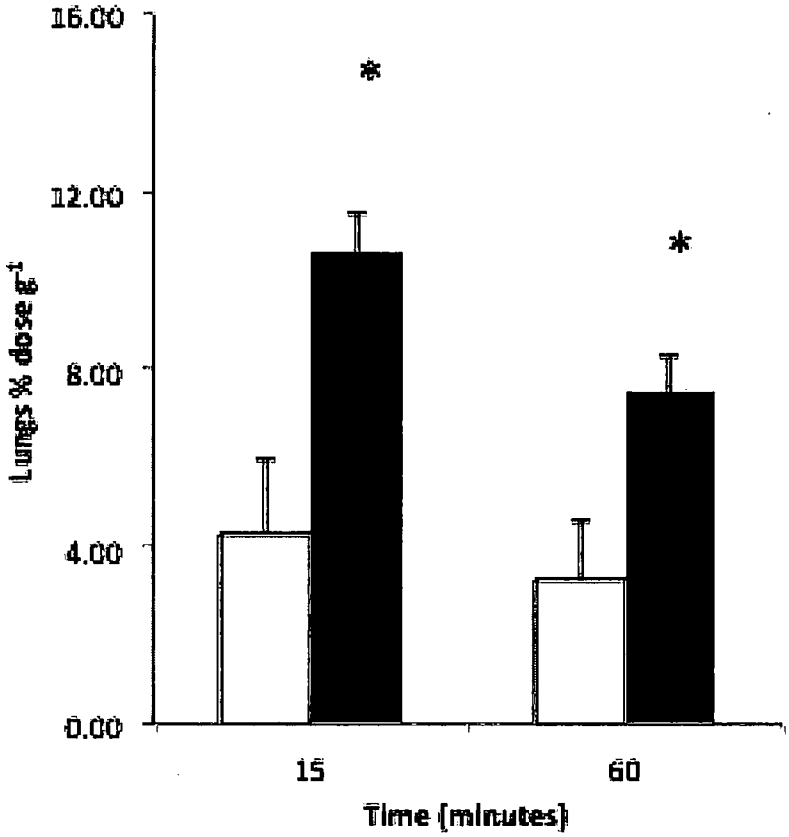


Figure 7

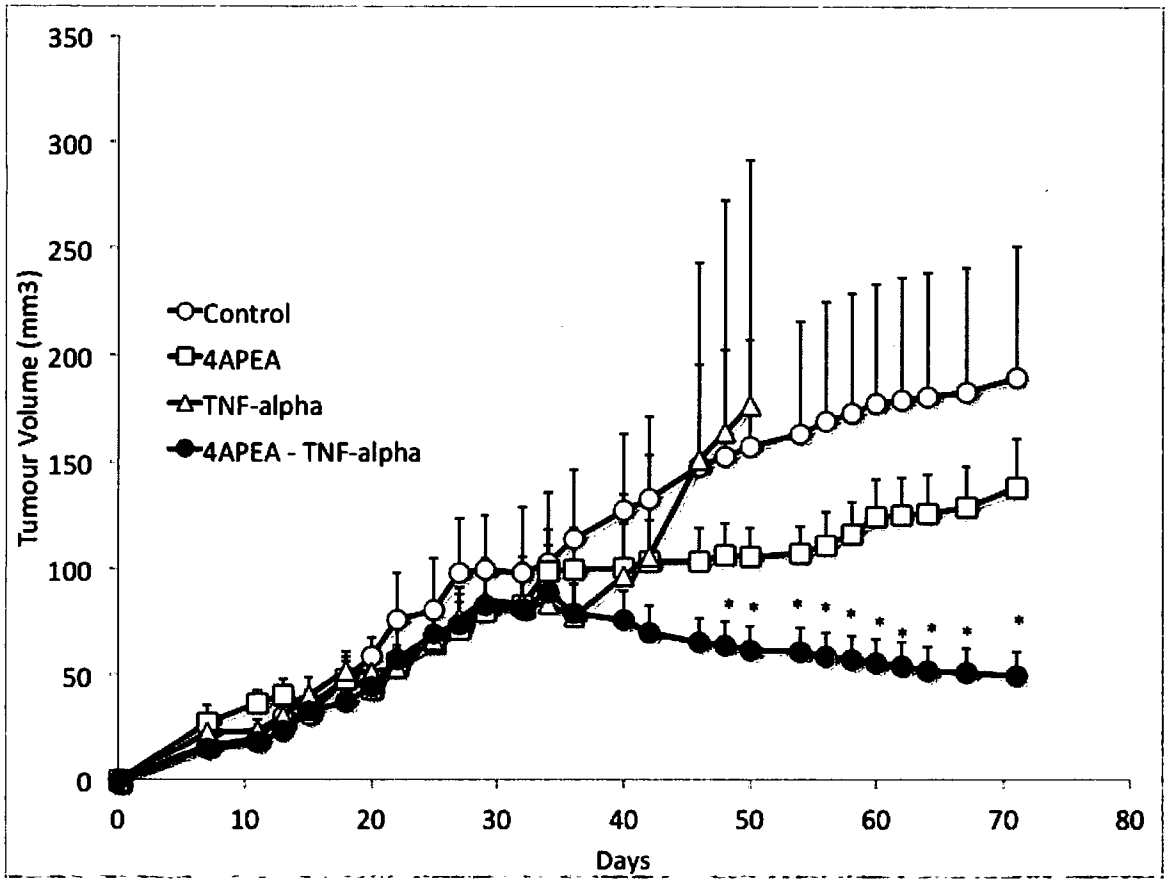

350
300
250
200
150
100
50
0
Tumour Volume (mm3)
Control
4APEA
TNF-alpha
4APEA - TNF-alpha
0
10
20
30
40
50
60
70
80
Days

Figure 8

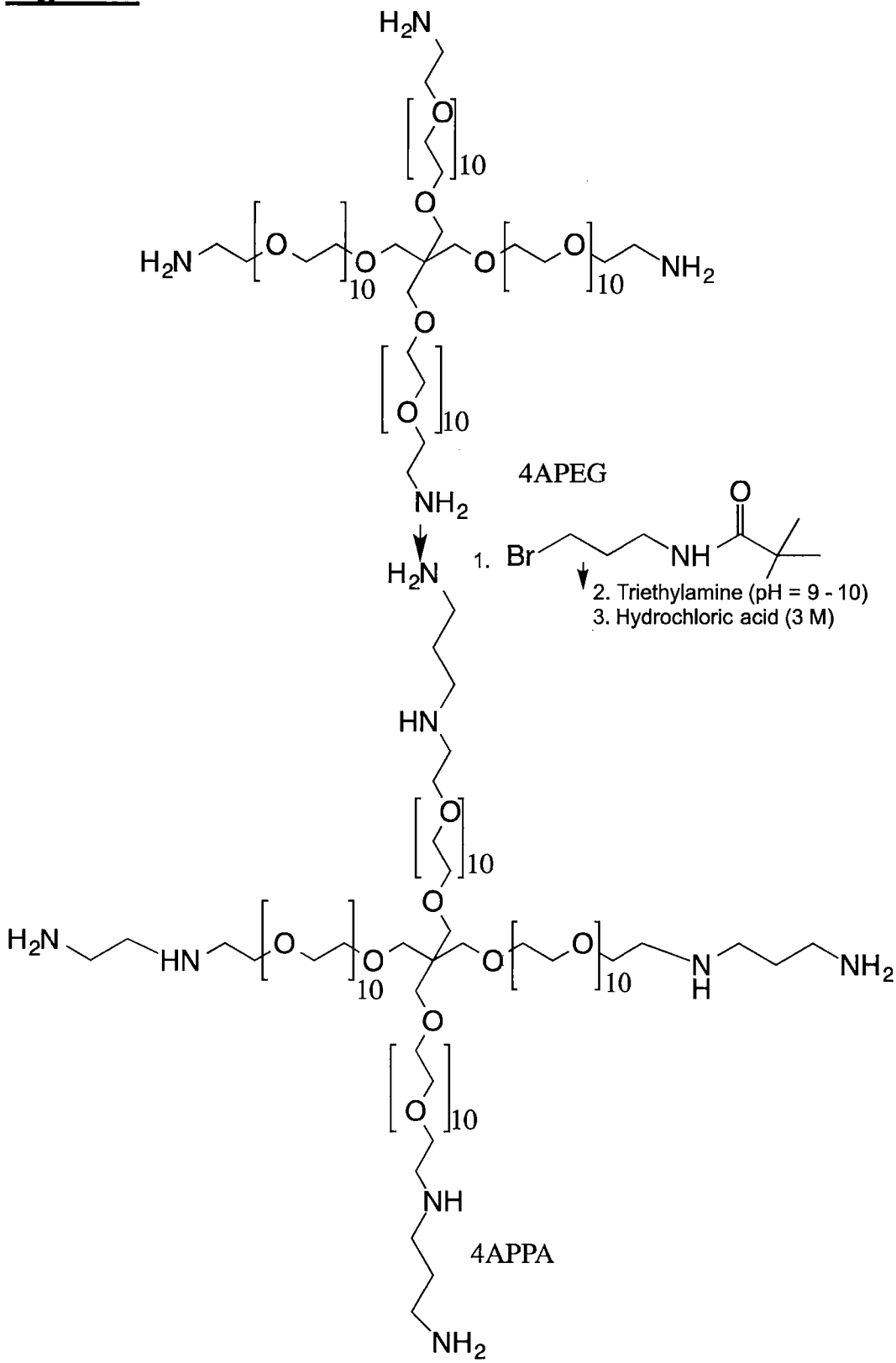

4APEG
1. Br
NH
2. Triethylamine (pH = 9 - 10)
3. Hydrochloric acid (3 M)
4APPA

Figure 9

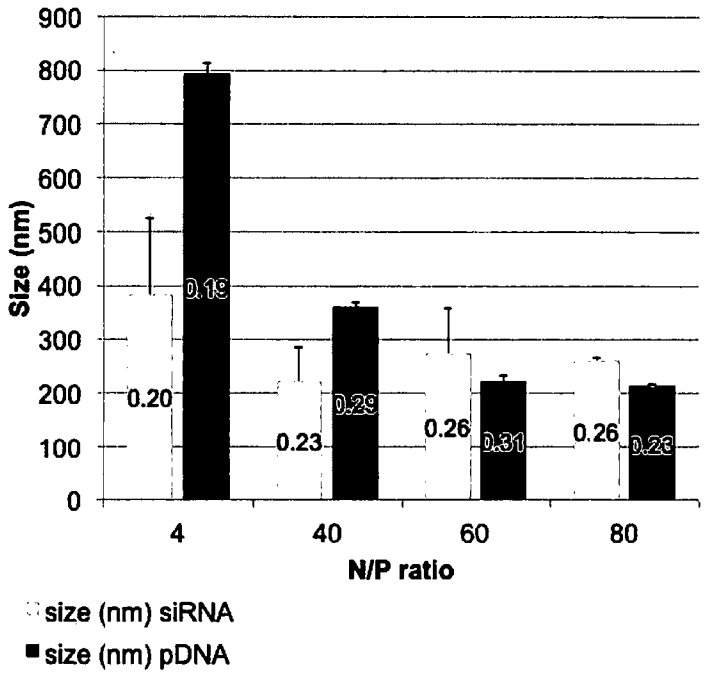


Figure 10

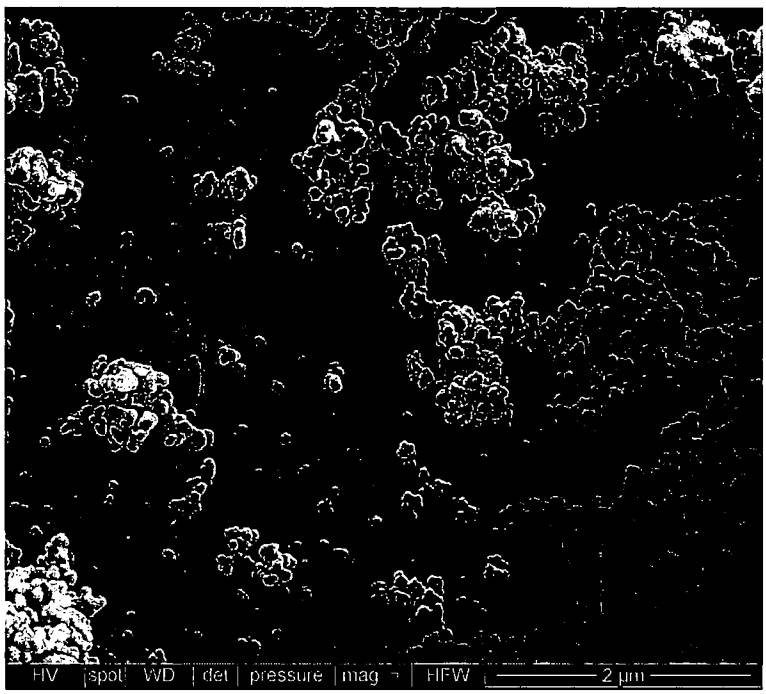


Figure 11

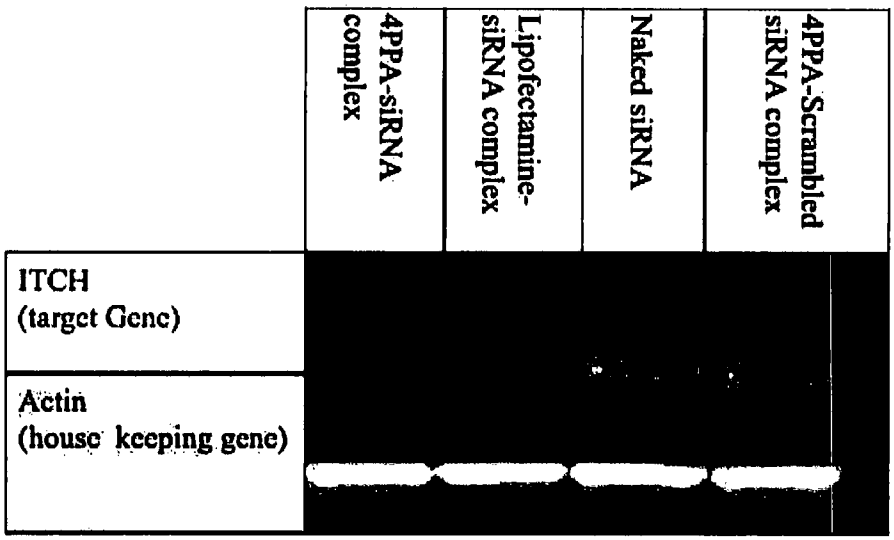


Figure 12

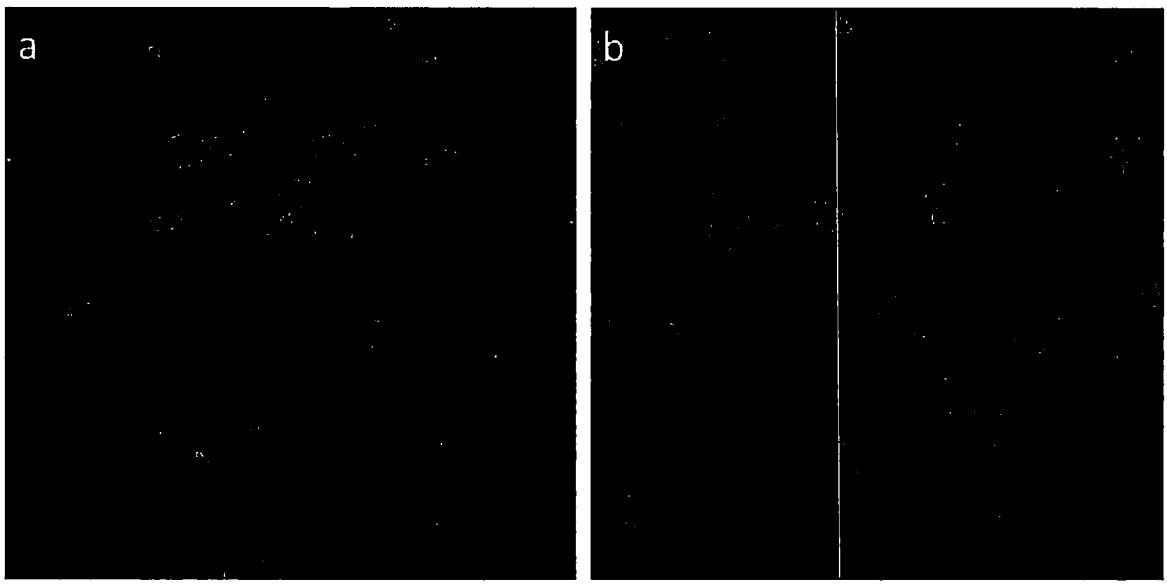
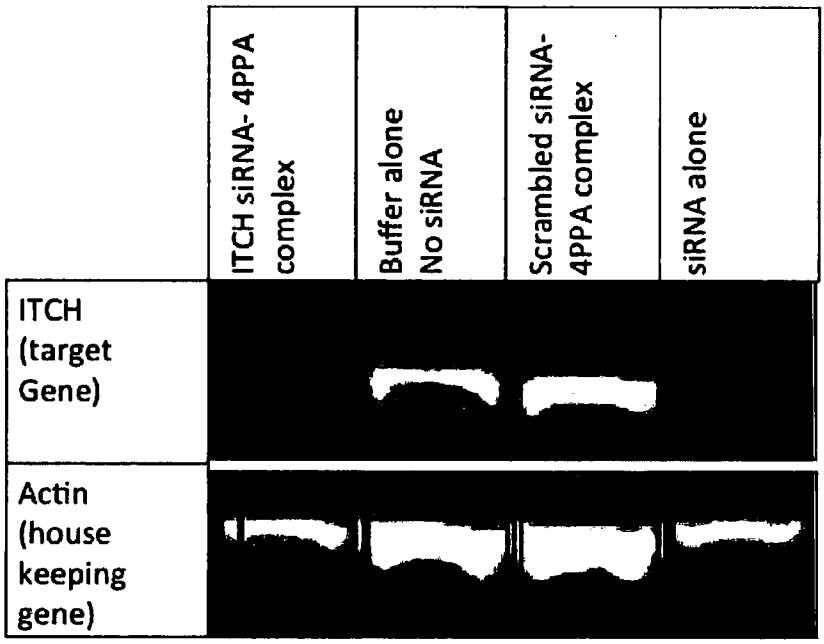


Figure 13


ITCH siRNA- 4PPA complex
Buffer alone No siRNA
Scrambled siRNA-4PPA complex
siRNA alone
ITCH (target Gene)
Actin (house keeping gene)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/051193

A. CLASSIFICATION OF SUBJECT MATTER
INV. C08G65/333 C08G65/337
ADD.

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)
C08G

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IEMSAM-ARNG J. ET AL.: "Star shaped polyethylene yield biocompatible gene delivery systems", PHARMACEUTICAL NANOTECHNOLOGY, vol. 2, no. 4, 2014, pages 182-195, XP008180760, the whole document -----	1-24
X	WO 02/094901 A1 (DOW GLOBAL TECHNOLOGIES INC [US]) 28 November 2002 (2002-11-28) page 18, line 15 - line 20 -----	1-3
X	WO 2008/112952 A1 (HUNTSMAN SPEC CHEM CORP [US]; BURTON BRUCE L [US]; RENKEN TERRY L [US]) 18 September 2008 (2008-09-18) claims 1-3 -----	1-3
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2016	28/07/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	O'Sullivan, Timothy

3

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INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/051193

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ALBERTAZZI L. ET AL: "Enhanced bioactivity of internally functionalised cationic dendrimers with PEG cores", BIOMACROMOLECULES, vol. 13, 2012, pages 4089-4097, XP002759460, table 1 Scheme 1	1-24
A	WO 2010/111594 A1 (DU PONT [US]; CHENAULT HENRY KEITH [US]) 30 September 2010 (2010-09-30) page 16 - page 18; claims 1,11	1-24
A	A. BÜCKMANN ET AL: "Functionalization of poly(ethylene glycol) monomethoxy-polyethylene glycol", MAKROMOL. CHEMIE, vol. 182, 1981, pages 1379-1384, XP002759461, scheme 1; compounds 2a,6a	1-24

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2016/051193

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2010111594 A1	30-09-2010	CN 102365101 A	29-02-2012
		EP 2411062 A1	01-02-2012
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		US 2012014909 A1	19-01-2012
		WO 2010111594 A1	30-09-2010