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(71) Applicant *(for all designated States except US)*: **ABBOTT LABORATORIES** [US/US]; Dept. 377 Bldg AP6A-1, 100 Abbott Park Road, Abbott Park, Illinois 60064-6008 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **TARCHA, Peter** [US/US]; 21651 Gelden Road, Lake Villa, Illinois 60046 (US). **MERDAN, Thomas** [DE/US]; 112 Mckinley Avenue, Libertyville, Illinois 60048 (US). **WAGNER, Ernst** [AT/DE]; Guardinistr 94, D-81375 München (DE). **KLÖCKNER, Julia** [DE/DE]; Arnimstr 1, D-81369 München (DE).

(74) Agents: **REININGER, Irene, M.** et al.; Dept. 377 Bldg AP6A-1, 100 Abbott Park Road, Abbott Park, Illinois 60064-6008 (US).

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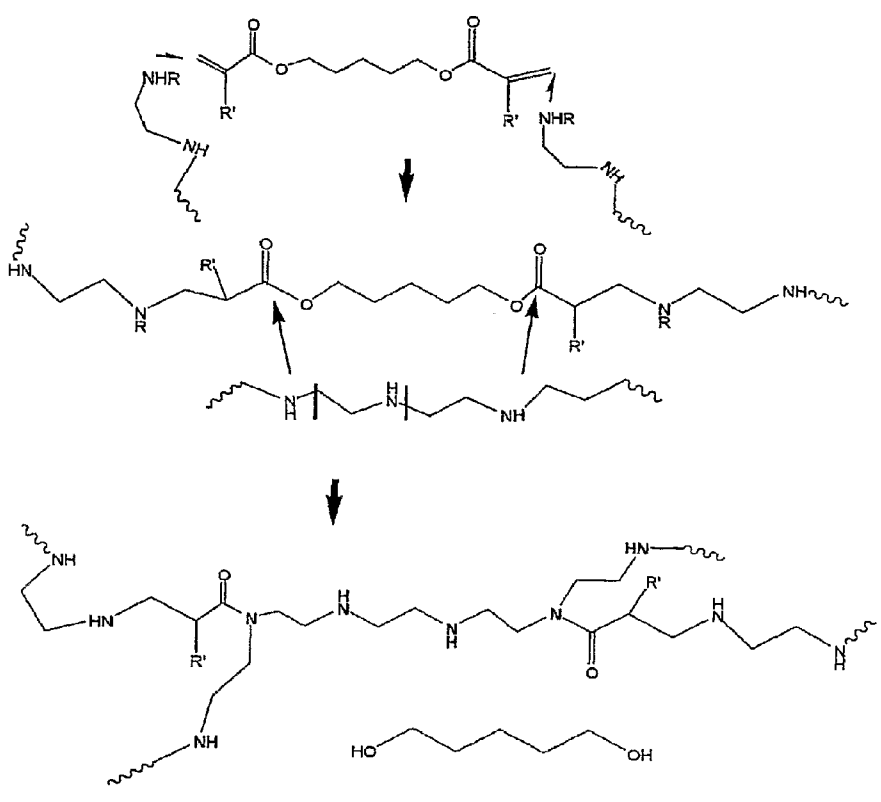

R & R' = H or alkyl
R & R' = H ou alkyle

(57) Abstract: The present invention provides a unique non-viral carrier for nucleic acid delivery *in vitro* and *in vivo*, and methods of using thereof.

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CHEMICALLY MODIFIED POLYCATION POLYMER FOR siRNA DELIVERY

This application claims the benefit of U.S. Provisional Patent Application Nos. 60/761,182, filed Jan. 23, 2006 and 60/787,057, filed March 29, 2006, the disclosures of each of which are hereby incorporated by reference in their entirety for all purposes.

BACKGROUND OF THE INVENTION

Non-viral delivery systems for genes have received increasing attention due to the growing implementation of human gene therapy. Cationic lipids formulated into liposomes, and soluble cationic polymers have been demonstrated to readily complex nucleic acid-based drugs and effectively deliver them into cells *in vitro*. The major barrier on the cellular level is the endosomal membrane, which can be overcome by cationic lipids via a flip-flop mechanism (Xu *et al,* Biochemistry Vol. 35(18) page 5616 (1996)) or by cationic polymers via the so-called proton-sponge mechanism (Boussif, PNAS Vol. 92(16) page 7297 (1995)). The proton-sponge hypothesis states that during the pH drop in the endosome a polymer may act as a buffer, thus requiring more protons to reach the final pH of approximately 5. This leads to an increased influx of chloride counter ions as well as water, which eventually results in bursting of the vesicle and release of its content into the cytoplasm. Significant *in vitro* as well as *in vivo* toxicity is frequently associated with cationic polymers and lipids. On the cellular level the cationic charge leads to membrane damage and vectors may cause necrosis as well as apoptosis. On the *in vivo* level cationic charge leads to binding to cellular blood components such as erythrocytes and/or non-specific association with serum proteins as well as vessel endothelia. A very rapid clearance by the RES prevents the agent from reaching the intended anatomical sites for intervention. Strategies have been developed to address these undesirable properties, such as charge shielding with proteins and stealth molecules, such as PEG as well as the linkage of active targeting ligands for the intended cells for therapy.

One of the most acceptable and widely used gene delivery polymers is polyethylenimine (PEI). A degradable PEI derivative having many of the desired properties for gene delivery has been described (D. W. Pack, Bioconjugate Chemistry Vol. 14 page 934 (2003)), with a gene delivery activity 16-fold greater than nondegradable 25,000 molecular

weight PEI and with low toxicity. It was synthesized by using PEI 800 branched and reacting it with 1,6 hexanedioldiacrylate at a 1 to 1 molar ratio in a Michael fashion. The molecular weight obtained was about 30,000 and based on proton NMR, the structure had numerous biodegradable ester linkages. At pH 5, the half-life for degradation was 30 hours. In a related work partial acetylation of polyethyleneimine with acetic anhydride resulted in up to a 21-fold enhanced gene delivery activity without alteration of the cytotoxicity of the polymers (D. W. Pack Pharmaceutical Research Vol. 21 page 365 (2004)). Other forms of degradable polymer for the therapeutic delivery of polynucleotide-based drugs such as DNA have been obtained using a Michael addition of various amines to various diacrylates (Langer *et al.*, JACS Vol. 123 pages 8155-8156, (2001); JACS Vol. 122, pages 10761-10768, (2000)). In this case the final carrier products had ester bonds and no primary or secondary amines that could participate in an N-acylation reaction; the presence of ester linkages in the polymer backbone improves biodegradability.

Work by Klibanov *et al* (Pharmaceutical Res. Vol. 22, pages 373-380, (2005)) used two different cross-linking agents, namely, disuccinimidyl suberate (DSS) and ethylene glycol bis[succinimidylsuccinate (EGS) to link both 423-Da and 2kDa PEI into a higher molecular weight carrier for DNA. The cross-linking agent DSS, after reaction with the amines of PEI produces amide linkages and retains the aliphatic backbone, which originally linked the two amine-reactive groups (active esters) as part of the carrier's structure. This produced amide bonds and retained the aliphatic backbone as part of the structure. The use of EGS with PEI also produced amide linkages, but because of its structure, the carrier had ester linkages as well. Klibanov *et al* claimed that the material made with EGS was 30 times more efficient in gene delivery than that made with DSS. Klibanov *et al* suggested "the higher degradability of the EGS-based conjugate facilitates the release of DNA (vector unpacking) and hence enhances the transcriptional availability of DNA".

Ideally, an optimal non-viral carrier should be a robust polymer with low toxicity, and high gene delivery efficiency. A major advantage of the polymer of the present invention is the ability to deliver a wide range of nucleic acids. While standard polymers such as PEI 25 kDa are efficient in plasmid DNA delivery they are inefficient in delivering siRNAs and no substantial gene expression knockdown can be observed even at higher polymer doses (Kim *et al* Bioconjugate Chemistry Vol.17 pages 241-244, 2006). For linear PEI the literature is somewhat contradictory (Hassani *et al* J Gene Medicine, Vol. 7 pages 198-207, 2005; Urban-

Klein *et al* Gene Therapy Vol 12, 2005), however, data from the literature as well as experiments performed by applicants point out that linear PEI 22 kDa is suitable for knockdown of transiently transfected genes; however, it is not suitable to generate a robust knockdown in stably transfected cell lines. In contrast to these standard reagents the polymer of the present invention is capable of achieving knockdown in transiently as well as stably transfected cell lines and exhibits a high efficiency in plasmid delivery

In summary, there is a need for a nonviral nucleic acid carrier system with the advantages of increased stability as a commercial product, low toxicity, transfection efficiency, the potential ability for elimination by the kidney if the molecular weight is kept low, biodegradability through the amide linkages, and numerous primary and secondary amines through which targeting and stealth ligands may be chemically attached.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a compound having the general Formula I:

$$\left[[\text{polycation}]_a - [L]_b - [\text{polycation}]_c \right]_d$$

wherein:

polycation is a polyethylenimine;

L is an non-ester linker moeity;

a is an integer in the range of about 1 to about 20;

b is an integer in the range of about 1 to about 10;

c is an integer in the range of about 1 to about 20; and

d is an integer in the range of about 1 to about 1000.

Preferably the PEI is oliogethyleneimine (OEI). The polycations of Formula I can optionally have the same polycation recurring or may also have a combination of varying polycations recurring.

"L" or linker of Formula I is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a succinamidyl linker moiety and combinations thereof. The linker moiety is bonded to an amine group contained within the polycation. In a preferred embodiment, the non-ester containing linker moiety is a propionyl unit defined as the chemical group

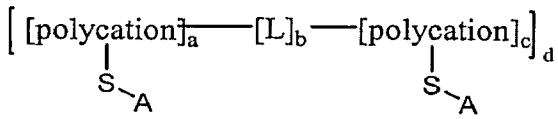
represented by: $-CH_2-CHR'-CO-N-$ where R' is H or an alkyl group. In a more preferred embodiment, the non-ester linker is a beta-aminopropionylamide linker moiety.

In another embodiment, the compound of Formula I further comprises a biomolecule that is complexed to the compound. The biomolecule may bear one or more an anionic groups and may form an ionic bond with the compound of Formulas I or Ia. Examples of biomolecules bearing one or more anionic groups include nucleic acids (e.g., DNA, single strand RNA, double strand RNA, ribozyme, DNA-RNA bybridizer, siRNA, anitosence DNA and antisence ligo), proteins, peptides, lipids and carbohydrates.

Yet a further embodiment provides a method of transfecting a eukaryotic cell, comprising contacting the cell with such a compound of Formula I and a biomolecule, to thereby deliver the biomolecule to the cell. The method may involve treating a mammal, comprising identifying a mammal in need of gene therapy and administering such a compound to the mammal. In a preferred embodiment, the biomolecule is siRNA, wherein the siRNA is effective to lower expression of a gene of interest.

Another embodiment provides a pharmaceutical composition comprising a compound of Formula I and a biomolecule.

Another embodiment further provides the compound having of general Formula Ia:



wherein:

polycation is a polyethylenimine;

L a non-ester linker moiety;

S is a spacer or is absent;

A is an agent or is absent;

a is an integer in the range of about 1 to about 20;

b is an integer in the range of about 1 to about 10;

c is an integer in the range of about 1 to about 20; and

d is an integer in the range of about 1 to about 1000.

L or linker of Formula I is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a succinamidyl linker moiety and combinations thereof. The linker moiety is

bonded to an amine group contained within the polycation. In a preferred embodiment, the non-ester containing linker moiety is a propionyl unit defined as the chemical group represented by: -CH_2-CHR'-CO-N- where R' is H or an alkyl group. In a more preferred embodiment, the non-ester linker is a beta-aminopropionylamide linker moiety.

S is a spacer or is absent. The spacer can be, for example, a substituted or unsubstituted, saturated or unsaturated hydrocarbon chain and a substituted or unsubstituted, saturated or unsaturated hydrocarbon chain interrupted by at least one heteroatom such as oxygen, nitrogen and sulfur. Preferably, the hydrocarbon chain comprises 2-20 carbon atoms, more preferably 2-10 carbon atoms and most preferably 2-6 carbon atoms. Suitable spacers may also include but are not limited to polyethylene glycol (PEG).

A is an agent that may facilitate one or more functions in the eukaryotic cell, e.g., receptor recognition, internalization, escape of the biomolecule from cell endosome, nucleus localization, biomolecule release, and system stabilization. The therapeutic agents may include, but not limited to cytotoxic agents, such as paclitaxel, endosomolytic agents, hydrophobic polymers, including but not limited to benzoyl and lauryl groups, targeting moieties, and shielding agents. The shielding agent may include but is not limited to hydrophilic entities, comprising but are not limited to polyethylene glycol (PEG), lactose, sugar, and polyacrylaminde. Targeting ligands include, but are not limited to transferrin, epidermal growth factor, folate, peptides, antibodies or fragments thereof, sugars, and integrin-binding entities such as RGD peptides.

In another embodiment, the compound of Formula Ia further comprises a biomolecule that is complexed to the compound. The biomolecule may bear one or more an anionic groups and may form an ionic bond with the compound of Formula Ia. Examples of biomolecules bearing one or more anionic groups include nucleic acids (e.g., DNA, single strand RNA, double strand RNA, ribozyme, DNA-RNA bybridizer, siRNA, anitosence DNA and antisence ligo), proteins, peptides, lipids and carbohydrates.

In another embodiment, the compounds of Formula Ia further comprise a biomolecule, and an agent (i.e., a shielding, targeting and/or delivery enhancing agent) that is complexed to the compound, optionally including a spacer.

Yet a further embodiment provides a method of transfecting a eukaryotic cell, comprising contacting the cell with such a compound of Formula Ia and a biomolecule, optionally further comprising an agent to thereby deliver the biomolecule to the cell. The

method may involve treating a mammal, comprising identifying a mammal in need of gene therapy and administering such a compound to the mammal. In a preferred embodiment, the biomolecule is siRNA, wherein the siRNA is effective to lower expression of a gene of interest.

Another embodiment provides a pharmaceutical composition comprising a compound of Formula Ia and a biomolecule, and may further comprise an agent that is complexed to the polymer.

Yet a further embodiment provides a method of treating a mammal, comprising identifying a mammal in need of gene therapy and administering the compound of Formula I complexes with a biomolecule to a mammal, wherein said biomolecule is siRNA that is effective to lower expression of a gene of interest.

Yet a further embodiment provides a method of treating a mammal, comprising identifying a mammal in need of gene therapy and administering the compound of Formula Ia complexes with a biomolecule to a mammal, wherein said biomolecule is siRNA that is effective to lower expression of a gene of interest.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1a-1b. Proposed mechanism of polycation modification.

Figure 2. Infrared spectra for OEI-HD product.

Figure 3a-3b. Structural elements and IR for PEI-800 modified with suberic acid chloride.

Figure 4. siRNA delivery HUH7/EGPLuc cells using OEI-HD-1.

Figure 5. siRNA knockdown with OEI-HD-1 in different media.

Figure 6a-6b. siRNA knockdown with OEI-HD-1 in media containing serum.

Figure 7. Failed siRNA knockdown with OEI-SUB-1.

Figure 8. Results of *in vivo* use of chemically modified polycation for RAN-siRNA.

Figure 9a-9b. Beta-aminopropionylamide linker examples.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a unique nonviral carrier for biomolecule delivery, wherein the carrier is a polymer having polycations chemically linked by propionylamide units. The present invention further relates to compounds of Formulas I and Ia described below, methods of preparing said compounds, as well as method of using the compounds of Formulas I and Ia.

I. Compound of Formula I

An embodiment provides polycations chemically linked by proprionylamide units as described in Formula I:

$$[[\text{polycation}]_a\text{–}[L]_b\text{-}[\text{polycation}]_c]_d$$

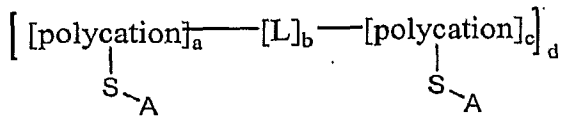
In Formula I, the polycation is defined as a molecule capable of obtaining more than two cationic charge when placed into aqueous solution. For example, in certain embodiments the polycation of Formula I may include, but are not limited to, polyethylenimine 400 Da – 750 kDa, dendrimer structures (e.g. polypropyleneimine dendrimers or PAMAM dendrimers with different structures and molecular weight), spermine, spermidine, triethylentetramine, tetraethylenpentamine, and pentaethylenhexamine. Preferably the polycation of the Formula I is a poly-ethyleneimine (PEI) and most preferably the polycation of Formula I is oliogethyleneimine (OEI). The polycations of Formula I can optionally have the same polycation recurring or may also have a combination of varying polycations recurring.

"L" or linker of Formula I is a non-ester containing linker moiety. Suitable non-ester containing linkers include, but are not limited to, an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a succinamidyl linker moiety and combinations thereof. The linker moiety is bonded to an amine group contained within the polycation. In a preferred embodiment, the non-ester containing linker moiety is a propionyl unit defined as the chemical group represented by: $-CH_2-CHR'-CO-N-$ where R' is H or an alkyl group. In a more preferred embodiment, the non-ester linker is a beta-aminopropionylamide linker moiety.

The polycation may contain recurring units of the same polycation or a combination of varying polycations, in which a and c are integers in the range of about 1 to about 20. Moreover, L can be recurring, also with either the same linker moiety or with a combination of varying linker moieties and therefore b is an integer in the range of about 1 to about 10. The entire compound of Formula I one can also be recurring and d is an integer in the range of about 1 to about 100 and is preferably in the range of about 30.

II. Compound of Formula Ia

A further embodiment provides polycations chemically linked by proprionylamide units as described in Formula Ia:



wherein:

"S" is a spacer or is absent. The spacer can be, for example, a substituted or ussubstituted, saturated or unsaturated hydrocarbon chain and a substituted or unsubstituted, saturated or unsaturated hydrocarbon chain interrupted by at least one heteroatom such as oxygen, nitrogen and sulfur. Preferably, the hydrocarbond chain comprises 2-20 carbon atoms, more preferably 2-10 carbon atoms and most preferably 2-6 carbon atoms. Suitable spacers may also include but are not limited to polyethylene glycol (PEG).

In a further embodiment of compounds of Formula Ia the may also include an agent ("A"). "A" is an agent or is absent. Preferably, "A" is an agent that may facilitate one or more functions in the eukaryotic cell, e.g., receptor recognition, internalization, escape of the biomolecule from cell endosome, nucleus localization, biomolecule release, and system stabilization. The therapeutic agents may include, but not limited to cytotoxic agents, such as paclitaxel, endosomolytic agents, hydrophobic polymers, including but not limited to benzoyl and lauryl groups, targeting moieties, and shielding agents. The shielding agent may include but is not limited to hydrophilic entities, comprising but are not limited to polyethylene glycol (PEG), lactose, sugar, and polyacrylaminde. Targeting ligands include, but are not limited to transferrin, epidermal growth factor, folate, antibodies or fragments thereof, peptides, sugars, and integrin-binding entities such as RGD peptides.

It is understood that at least on of S and A must be present in Formula Ia.

The polycation may contain recurring units of the same polycation or a combination of varying polycations, in which a and c are integers in the range of about 1 to about 20. Moreover, L can be recurring, also with either the same linker moiety or with a combination of varying linker moieties and therefore b is an integer in the range of about 1 to about 10. The entire compound of Formula Ia one can also be recurring and d is an integer in the range of about 1 to about 100 and is preferably in the range of about 30.

The molecular weight of the compound of Formula I may range from about 800 Daltons to about 1,000,000 Daltons, preferably in the range of about 20,000 Daltons to about 200,000 Daltons, and most preferably in the range of about 20,000 Daltons to about 30,000.

The molecular weight of the compound of Formula Ia may range from about 800 Daltons to about 1,000,000 Daltons, preferably in the range of about 20,000 Daltons to about 200,000 Daltons, and most preferably in the range of about 20,000 Daltons to about 30,000.

The molar ration of polycation to L is 20-50. While the molar ration of free amines on the polycation to agents can vary depending on agent and may be from about 1000 and 2.

III. The Compounds of Formula I or Ia in Complex with Biomolecules

The compound of Formula I may form complexes with biomolecules and thus are useful as carriers for the delivery of biomolecules to cells. Examples of biomolecules that form complexes with the compound of the Formula I include nucleic acids, proteins, peptides, lipids, and carbohydrates. Examples of nucleic acids include DNA, single strand RNA, double strand RNA, ribozyme, DNA-RNA hybridizer, and antisense DNA, e.g., antisense oligo. A preferred nucleic acid is siRNA. Cationic lipopolymers that comprise a biomolecule that is complexed to the polymer may be formed by intermixing the cationic lipopolymers and biomolecules in a mutual solvent, more preferably by the methods described in the examples below.

IV. The Compounds of Formula I or Ia in Complex with Biomolecules and Optionally with Agents

The polymer of the present invention can also form an ionic complex or covalent bond with specific therapeutic agents, including but not limited to cytotoxic agents, such as paclitaxel, endosomolytic agents, hydrophobic polymers and other targeting moieties.

The OEI-HD carrier can be modified with shielding ligands, which will reduce the occurrence of unwanted non-specific interactions after in vivo administration, and therefore improve circulation lifetime after administration. Shielding ligands comprise hydrophilic entities, comprising but are not limited to polyethylene glycol (PEG), lactose, sugar, and polyacrylaminde. Additionally, to improve uptake into the tissue of interest, the carrier, which can be OEI-HD or shielded OEI-HD, can have targeting ligands conjugated to the same. Examples of targeting ligands comprise but are not limited to transferrin, epidermal growth factor, folate, antibodies or fragments thereof, sugars, and integrin-binding entities such as RGD peptides.

V. The Preparation of the Compounds of Formula I and Ia

The cross-linking of the polycation, preferably a poly-ethyleneimine (PEI), occurs by the Michael addition of a fraction of the polymer's amines to vinylic groups of cross-linking

groups and from N-acylation of pendant ester groups. Cross-linking groups, can be acrylate or methacrylate ester monomers. It is understood that acrylate and methacrylate ester monomers comprise several groups including but not limited to diacrylate, dialkylacrylate, dimethacrylate, diacrylate ester monomers. Acylation is defined as the introduction of an acyl group into the molecule of an organic compound having hydroxyl (O-acylation) or amino (N-acylation) groups. Additional N-acylation of the polymer can be achieved by the reaction of esters, such as ethyl acetate and anhydrides with the polymer. The chemical modifications may occur at both the primary and secondary amines of the polymer structure, thus reducing the net number of ionizable groups. If multifunctional reactants are used for the modification, an increase in the average molecular weight of the polymer occurs. These mechanisms and chemical units introduced are illustrated in Fig. 1.

The resulting polymer has application as a nonviral synthetic carrier for a variety of entities with opposite charge, including but not limited to nucleic acid and therapeutic peptides. A major advantage of the polymer of the present invention is the ability to deliver a wide range of nucleic acids. While standard polymers such as PEI 25 kDa are efficient in plasmid DNA delivery, they are inefficient in delivering siRNAs and no substantial gene expression knockdown can be observed even at higher polymer doses (Kim *et al* Bioconjugate Chemistry Vol. 17, pages 241-244, 2006). For linear PEI the literature is somewhat contradictory (Hassani *et al* J Gene Medicine Vol 7, pages 198-207, 2005; Urban-Klein *et al* Gene Therapy Vol 12, 2005), however data from the literature as well as experiments by applications point out that linear PEI 22 kDa is suitable for knockdown of transiently transfected genes, however it is not suitable to generate a robust knockdown in stably transfected cell lines. In contrast to these standard reagents the polymer of the present invention is capable of achieving knockdown in transiently as well as stably transfected cell lines and exhibits a high efficiency in plasmid delivery. Therefore double stranded RNA molecules such as small interfering RNAs (siRNA) are uniquely suitable for delivery with the polymer carrier of the present invention. The siRNA delivery can be for therapeutic purposes or for target validation, i.e. identification of potential targets for novel therapeutic purposes.

The polymer carrier of this invention can be prepared from various oligoamines including but not limited to dendrimer structures (e.g. polypropyleneimine dendrimers or PAMAM dendrimers with different structures and molecular weights), spermine, spermidine, triethylentetramine, tetraethylenpentamine, and pentaethylenhexamine or from a base

material consisting of branched polyethylene imine, with a 400 to 25,000 MW range. The branched polyethylene imine is chemically modified or cross-linked with mono- bi- or multi functional agents. The polyethylene imine contains a plethora of primary, secondary and tertiary amines and those amines make up approximately 30 % of the polymer mass. Both the primary and secondary amines are available for reaction with the cross-linking agent. A crosslinking agent is defined as a molecule that has at least 2 reactive groups and is used to chemically link at least 2 polymer molecules. Agents that can be used as a cross-linking agent include but are not limited to ethylene glycol diacrylate, ethylene glycol dimethacrylate, 1,6 hexanediol diacrylate, polyethylene glycol 600 diacrylate and other di- or multiacrylate or di-or mult-methcrylate molecules. The preferred agent used in this invention is a diacrylate, namely 1,6 hexanediol diacrylate abbreviated as HD. HD has four possible reactive sites, namely 2 vinylic groups and 2 ester goups. Since the polyethyleneimine is of low molecular weight we commonly refer to it as oliogethyleneimine, abbreviated as OEI. When the OEI is combined with the HD and heated for several days, the reactive amines add across the two sites of unsaturation in a Michael fashion.

Normally the ratio of HD to OEI used is one-to-one, on a molar basis. When the ratio is more than one-to-one, then the numerical designation reflects the molar ratio, for example OEI-HD-1 means that a HD to OEI ratio is one-to-one, OEI-HD-5 means that a HD to OEI ratio is five-to-one. When the reaction is complete, proton NMR shows that all of the vinylic groups have been consumed and the resulting product is usually soluble in water. So crosslinking of the OEI chains has not occurred to an extent that a swellable hydrogel has formed. Size exclusion chromatography of a typical sample gave a chromatogram which allowed the calculation of the number and weight average molecular weight and hence the polydispersity (weight average molecular weight divided by number average molecular weight or Mw/Mn). These values were 3000, 16,000 and 5.3, respectively. A polydispersity value of 1 indicates a monodisperse molecular weight. Value of 2 – 3 are somewhat narrow whereas 4 and above indicate a broader distribution.

This polymer was designed to be less toxic than higher molecular weight polyethyleneimine, (PEI) 25,000, the levels of which are unacceptable for in vivo use in gene transfection or siRNA delivery. Low molecular weight PEI's, like OEI 800, used as a starting material in this invention, is relatively non toxic, but not very effective at delivering nucleic acids across a cell membrane. The product from the reaction described above is both

relatively non toxic to cells and very effective at delivering siRNA and DNA across cell membranes. It also releases them into the cytoplasm, so they can perform a biological effect in the cell. An additional feature of the idealized structure shown above is that it should be biodegradable by means of the ester linkages, which can be part of the structure. In theory, hexanediol could be obtained upon ester hydrolysis as a by-product as well as OEI 800 containing 1-alkylamino-propanoic acid end groups.

Infrared spectra were run on such carriers after synthesis and isolation, but we were surprised to find that very little ester was present, and a strong amide peak was dominant. Carboxylic acid was not indicated in the infrared spectrum either, which seemed to rule out premature ester hydrolysis (Figure 2). Proton NMR of the product after dialysis against distilled water indicated an absence of aliphatic methylene groups which should be part of the HD linkages. A reaction that can explain these results is the acylation of the ester bonds by residual amines on the OEI, which should be in abundance based on the molar ratio of starting materials. Such acylation would also provide a by-product of 1,6 hexanediol, which is water soluble and removable by dialysis. Further evidence of ester acylation by amines on the OEI was provided by washing of the product with ethyl acetate, a non-solvent for the polymer. In this case analysis indicated that a limited amount of N-acylation of ethyacetate occurred.

A similar polycation carrier can be obtained by a two-step process. In this alternative two-step process, the first step consist in the core polycation being modified with a crosslinking agent, and the second step consist in a further modification by addition of a similar or different type of polycation, for example, spermine and pentaethylenhexamine.

EXAMPLES

The present invention will be further clarified by the following examples, which are only intended to illustrate the present invention and are not intended to limit the scope of the present invention.

Example 1. Synthesis of the carrier, OEI-HD-1.

5.0 g (0.0063 moles) of polyethylene imine (weight average molecular weight 800) were dissolved in 7.5 ml of DMSO. In a separate container, 3.3 ml of DMSO and 1.4 ml = 1.4 g (0.0063 moles) 1,6 hexanediol diacrylate were added. Both solutions were mixed. In a

50 ml round bottom flask immersed in oil bath thermostated at 60 degrees C and fitted with a magnetic stir bar. The flask was loosely stoppered and allowed to react for 4 days. Then the reaction solution was added dropwise to 200 ml of a rapidly stirred solution of ethyl acetate whereby a viscous material formed on the bottom and sides of the flask. The solvent was decanted off and a fresh 200 ml aliquot of ethyl acetate was added and the materials mixed. This was decanted again and an additional 100 ml aliquot was added, mixed and decanted leaving behind the viscous material. The material was transferred to a boat made from aluminum foil and it was placed in a vacuum oven at room temperature overnight. This evacuation process fails to remove all of the ethyl acetate because of the low surface area and high viscosity of the material and required further purification.

Example 2. Purification of OEI-HD-1 by Dialysis

Weighted out 0.80g of OEI-HD-1 and added it to a scintillation vial followed by 10 ml of Dulbucceos PBS buffer. It dissolved after a short time with shaking. Preconditioned about 1 linear foot of Spectrum 3500 cut-off dialysis membrane (0.4 ml / cm of length capacity) by boiling it in a beaker of distilled water for about 10 minutes. Then a knot was tied in one end of the dialysis tubing and the OEI-HD-1 solution was added and sealed by tying a knot in the other end. The tube was placed in approximately 3 gallons of distilled water and the water was stirred gently for 4 days. After that the material was removed from the tubing and freeze dried yielding about 30 percent of the polymer mass that was added to the tubing. Proton and Carbon 13 NMR were run on this product.

Example 3. Precipitation of OEI-HD-1 into Dioxane.

Example 1 is repeated but instead of using ethyl acetate for washing, dioxane was substituted. The use of dioxane avoids the possibility of acetylation of free amines by the ethyl acetate ester.

Example 4. Synthesis of OEI-HD-5

Dissolved 5.0 g (0.0063 moles) of polyethylene imine (weight average molecular weight 800) in 7.5 ml of DMSO in a 125 ml glass bottle. To a separate container, added 17 ml of DMSO and 7.0 ml = 7.0g (0.0315 moles) 1,6 hexanediol diacrylate and mixed. Combined both solutions in the 125 ml bottle, loosely capped it and placed it in a oven

thermostated at 60 degrees C. After about 30 minutes into the reaction, a gel was seen to form. The reaction was allowed to continue for a total of 3 days. Then the DMSO was decanted from the gel and the gel was broken up into chunks with a spatula. A small chunk was placed in a 20 ml scintillation vial with about 10 ml of water whereupon most of the gel appeared to dissolve.

Example 5. Synthesis of OEI-HD-10

Dissolved 5.0 g (0.0063 moles) of polyethylene imine (weight average molecular weight 800) in 7.5 ml of DMSO in a 125 ml glass bottle. To a separate container, added 34 ml of DMSO and 14 ml = 14 g (0.0630 moles) 1,6 hexanediol diacrylate and mixed. Combined both solutions in the 125 ml bottle, loosely capped it and placed it in a oven thermostated at 60 degrees C. After about 30 minutes into the reaction, a gel was seen to form. The reaction was allowed to continue for a total of 3 days. Then the DMSO was decanted from the gel and the gel was broken up into chunks with a spatula. A small chunk was placed in a 20 ml scintillation vial with about 10 ml of water and shaken for 2 days. None of the material dissolved.

Example 6. Synthesis of OEI-Sub-1

Weighed out 1.0 g of PEI-800, (0.0013 moles) and added 5 ml of DMSO that had been dried by standing over magnesium chloride. The PEI was not completely soluble, but formed a cloudy suspension. Pipetted 0.22 ml (0.26g, density 1.172, 0.0013 moles) of suberoyl chloride into 5 ml of dry DMSO. All glassware was flamed to remove moisture. Added the suberoyl solution dropwise to to the PEI solution/suspension at room temperature with shaking by hand. An insoluble gel-like material formed immediately. The gel was washed 1X with excess fresh dry DMSO followed by 2X washes with excess dioxane. The residual solvent was decanted off and the gel was left under house vacuum at room temperature for 19 hours. The material had a noticeable stench even after vacuum drying. A sample was submitted for IR (microscope) and proton NMR in D_2O. The proton NMR indicated the presence of residual DMSO and dioxane, in addition to the expected water. Removed 50 mg for a file sample and completely dissolved the remainder of the sample in about 10 ml of distilled water. The sample was placed in a dialysis tube (3500 molecular weight cut-off) and dialyzed against about 12 liters of distilled water with gentle stirring with

a magnetic stir bar for 5 days. The sample was divided into two vials and freeze dried. Approximately 100 mg of sample was recovered after freeze drying. Correcting for the 50 mg that was retained as an impure file sample, about 90% of the material was lost, most likely by passing through the dialysis membrane. The proposed structure of PEI-800-Sub-1 is shown in Figure 3a and the IR of the product is graphically represented in Figure 3b.

Example 7 SiRNA knockdown with OEI-HD-1

The results of siRNA delivery on HUH7/EGFPLuc cells using OEI-HD-1 are summarized in Figure 4. Transfections were performed in 96-well-plates using 5,000 cells/well in serum-free medium (OptiMEM). OEI-HD-1/siRNA formulations were prepared in 20µl HBS (20mM HEPES, 150mM NaCl) and added to 80µl of serum-free medium (100µl total volume). Four hours following delivery, transfection medium was replaced by growth medium and two days later luciferase activity was measured. Using 0.10µg siRNA (40nM) and the C/P ratio 8/1 (OEI-HD-1/siRNA: w/w) up to 50% knockdown of luciferase activity was achieve compared to transfection using unspecific MutsiRNA. With the purpose of clarification, C/P ratio means the carrier to plasmid weight ratio, which for the purpose of the present invention can be DNA or siRNA. The MutsiRNA is used as a control and is a good measure of the toxicity of the carrier. So if a reduced signal is seen with the MutsiRNA, which should have no biological activity, the knockdown seen with the specific siRNA at the same concentration should be corrected for the toxic effect of the carrier on the cells.

Using 0.25µg siRNA (100nM) and the C/P ratio 4/1 up to 60% knockdown of luciferase activity was achieved compared to transfection using unspecific MutsiRNA. For 0.50µg siRNA (200nM) up to 80% knockdown was observed. The use of higher concentration of siRNA (up to 1.00µg siRNA (400nM) did not lead to any further reduction of luciferase activity. Thus for the delivery in HUH7/EGFPLuc using OEI-HD-1 (complexes in HBS, transfection in serum-free medium) the maximal knockdown of luciferase expression can be achieved by 200nM (0.50µg siRNA per 5,000 cells) and the C/P ratio 2/1.

Example 8. SiRNA knockdown with OEI-HD-1 in Different Buffer Media

The ability of OEI-HD-1 to knockdown the luciferase expression was tested in different serum-free complexation media (HBS: 20mM HEPES, 150mM NaCl; HBG: 20mM HEPES, 5% glucose; OptiMEM: salt reduced serum-free medium, Gibco). Independent of the

complexation medium used, OEI-HD-1/siRNA formulations were able to knockdown of luciferase activity for up to 80% without significant differences between the complexation media. Formulations in OptiMEM were high efficient at 100nM (C/P: 2/1). This may be caused by the faster aggregation of OEI-HD-1/siRNA particles. Results are shown in Figure 5.

Example 9. SiRNA knockdown with OEI-HD-1 in Media Containing Serum

siRNA delivery in HUH7/EGFPLuc was performed using OEI-HD-1 in the presence of serum (10% FCS). OEI-HD-1/siRNA formulations were prepared in HBS (20µl) and complexes were added to 80µl of serum containing medium on the cells. Two different approaches were performed; first, medium was changed four hours following siRNA delivery as usually and second medium wasn't changed for two days. Following medium change, maximal knockdown of luciferase expression was achieved using 200nM siRNA and the C/P ratio 6/1, which was in contrast to the optimal transfer conditions achieved in serum-free medium: 200nM siRNA, C/P ratio 2/1.

Without medium change, the optimal transfer conditions for OEI-HD-1/siRNA delivery were the same as in the absence of serum (200nM siRNA, C/P ratio 2/1). Furthermore, without medium change, OEI-HD-1 was much more toxic and at the C/P ratio 6/1 and 200nM the cells died. Interestingly, w/o medium change, already 100nM siRNA and the C/P ratio 4/1 were high efficient for expression knockdown of the luciferase gene.

In summary, OEI-HD-1 vehicle was also efficient for siRNA delivery in the presence of serum. Similar to results in serum-free medium up to 80% knockdown of luciferase expression was observed also in the presence of 10% FCS. Furthermore, without medium change, already lower siRNA concentration was sufficient to achieve maximal effect. Results are summarized in Figures 6a and 6b.

Example 10. Failed SiRNA knockdown with OEI-Sub-1

OEI-Sub-1 synthesized using OEI (800 Da) and suberoyl acid ($C_6H_{12}O_2Cl_2$) as a cross linker (as described in Example 6) was tested for siRNA delivery on HUH7/EGFPLuc cells using different polymer/siRNA ratios. Transfection was performed in 96-well plates and 5,000 cells/well in triplicates using LucsiRNA (GL3) and MutsiRNA (IX) (Dharmacon). OEI-complexes were prepared in HBS and siRNA delivery was performed in serum-free

medium for 4 hours. Transfection medium was then replaced by growth medium and luciferase expression was measured two days following siRNA delivery. The amount of siRNA was varied from 0.1 to 0.50μg per 5,000 cells and OEI-Sub-1/siRNA ratio was varied as well; however, no silencing of luciferase gene expression was observed as shown in Figure 7.

Example 11. Coupling of K5 peptide mimetic to OEI-HD-1.

OEI-HD (2.8 mg) is dissolved in 1 mL of reaction buffer containing 150 mM sodium chloride and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES), pH 7.5. *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (80 μg) is added to this solution in 80 μL of 100% ethanol while stirring. The reaction is allowed to continue for 90 min. SPDP-activated OEI-HD is then purified by gel filtration using Sephadex G-25. In the second step, a 3-fold molar excess of K5 peptide bearing a cysteine on the N-terminus is added in the same buffer. The reaction is allowed to proceed for 12 h at room temperature and then purified by gel filtration using Sephadex G-25.

Example 12. Coupling of RGDC (cysteine terminated) peptide to OEI-HD-1

OEI-HD (2.8 mg) is dissolved in 1 mL of reaction buffer containing 150 mM sodium chloride and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) pH 7.5. *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (80 μg) is added to this solution in 80 μL of 100% ethanol while stirring. The reaction is allowed to continue for 90 min. SPDP-activated OEI is then purified by gel filtration using Sephadex G-25. In the second step, a 3-fold molar excess of RGDC peptide is added in the same buffer. The reaction is allowed to proceed for 12 h at room temperature and purified by gel filtration using Sephadex G-25.

Example 13. Coupling of antibody Fragment (Fab')

Using a procedure derived from Merdan *et al.* Bioconjugate Chemistry Vol. 14, pages 989 (2003), OEI-HD (2.8 mg) is dissolved in 1 mL of reaction buffer containing 150 mM sodium chloride and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) pH 7.5. *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (80 μg) is added to this solution in 80 μL of 100% ethanol while stirring. The reaction is allowed to continue for 90 min. SPDP-activated OEI is then purified by gel filtration using Sephadex G-25.

In the second step, a 1.4-fold molar excess of freshly reduced Fab' is added in the same buffer. The reaction is allowed to proceed for 12 h at room temperature and purification is performed by gradient ion exchange chromatography using 0.9% NaCl, 10 mM HEPES pH 7.4 as buffer A and 3 M NaCl, 10 mM HEPES pH 7.4 as buffer B and MacroPrep HighS (from Amersham Pharmacia).

Example 14. Coupling of polyethylenglycol to OEI-HD via HMDI activation

PEG monomethyl ether (0.5-20 kDa) is dissolved in anhydrous chloroform (200 g/L) and activated with a 10-fold excess of hexamethylene diisocyanate, HMDI at 60 ° C for 24 h. Unreacted HMDI is carefully removed by repetitive extraction with light petrol.Subsequently the reaction of the isocyanate-terminated PEG with the amino groups of OEI-HD is carried out in anhydrous chloroform at 60 ° C for 24 h. The degree of PEGylation can be adjusted by varying the ratio of activated PEG to OEI-HD. The reaction solution is precipitated in diethyl ether or other suitable non-solvent and the product is dried *in vacuo*.

Example 15. Coupling of polyethyleneglycol to OEI-HD via PEG-NHS

PEG-N-hydroxy succinimidyl esters of the desired molecular weight are dissolved in DMSO and added to an aqueous solution of OEI-HD-1. The reaction is stirred for 24 hours and the pegylated polymer is isolated by e.g. size exclusion chromatography.

Example 16. Coupling of a cyclic RGD peptide via PEG-spacer to form a PEG-shielded targetable carrier.

RGD is coupled to polyethylene glycol following a procedure published in Nucleic Acids Research, Vol 32 page 149, 2004. A chemical conjugate of polyethylene glycol with RGD (H-ACRGDMFGCA-OH) is synthesized first. This is done by oxidizing the two cysteine residues forming a cyclic 10-mer RGD peptide with a disulfide bridge. Then 60 mg of the cyclic peptide is dissolved in 600µl dimethyl sulfoxide (DMSO). Triethylamine (TEA) 8.54 µl, pre-dissolved in 20 µl of tetrahydrofuran, is added to the peptide under nitrogen. After stirring for 1 min, a solution of activated PEG, namely, NHS-PEG-VS (212 mg in THF: DMSO; 300 µl:100 µl) is added in one portion to react the n-hydroxysuccinimide (NHS) group on the PEG with the amino terminus of the peptide. The reaction mixture is stirred at room temperature for 4 h and quenched with trifluoroacetic acid (TFA) at

equivalence to the TEA. The RGD-PEG-VS is purified by dialysis against distilled water followed by lyophilization. In the second step, 100 mg (21.7 μmol) of the purified RGD-PEG-VS intermediate is dissolved in 1 ml of anhydrous DMSO. To this solution, six equivalents of TEA dissolved in 0.5 ml THF is added and mixed. An aliquot of 9.4 mg (218 μmol in terms of amines) of OEI-HD dissolved in dimethylformamide (0.5 ml) is added to this solution and stirred at room temperature for 12 h to cause Michael addition of amines on the OEI with the vinyl sulfone (VS) on the PEG. The product is purified as the TFA salt by HPLC.

Example 17. OEI-HD prodrug

Paclitaxel is coupled to one end of a polyethylene glycol molecule as described (Materials Research Innovations Vol. 9 pages 13-14, 2005). The other hydroxyl end of the PEG chain is reacted with trichloro-s-triazine to make an activated PEG end group. The activated PEG is combined with OEI-HD in water at pH 9.0 to allow coupling of the PEG-paclitaxel to the OEI-HD carrier. This OEI-HD-PEG-paclitaxel moiety is combined with other OEI-HD polymers having targeting ligands as well as specific siRNA against the cells of interest to form a polyplex. This polyplex has properties of delivering agents that interfere with cellular transcription (siRNA) as well as delivering small cytotoxic agents i.e, paclitaxel.

Example 18. *In vivo* use of chemically modified polycation for RAN-siRNA delivery.

RAN siRNA is a small interfering RNA directed against RAN GTPase. This enzyme is essential for most cells and knockdown of expression leads to toxic effects
Fifteen (15) mice were injected subcutaneously on the back with 1 million Neuro 2A tumor cells, resulting in a local tumor, which was allowed to grow to a size of 3 mm diameter as measured with calipers through the skin. The mice were divided into three groups of 5 animals each, namely, the therapeutic group, the control group, and the vehicle group. The therapeutic group received RAN-siRNA polyplexed to OEI-HD carrier at a weight ratio of 0.6 to 1 and the carrier contained 10 % by weight of OEI-HD –PEG-Tf. Transferrin (Tf) was covalently linked to the OEI-HD through a PEG spacer and served as a targeting agent, since the tumor of interest is known to express a receptor for transferrin. The control group received siCONTROL (a control siRNA that is biologically inactive), polyplexed to OEI-HD

at a weight ratio of 0.6 to 1 and the carrier contained 10 % by weight of OEI-HD–PEG-Tf. The last group received the buffer vehicle, hepes buffered glucose. Each mouse received three (3) 200-microliter injections given 3 days apart through the tail vein. Each therapeutic injection contained 35 micrograms of siRNA and each control injection contained 40 micrograms of siCONTROL. The vehicle group received 200 microliters of buffer only. The tumor size and the animal body weight was measured every day for all animals.

The mice were allowed to live for 8 days after the first injections were made. No weight loss attributable to the study was seen in any of the experimental groups. The tumor growth curves for the siControl and buffer arms were essentially the same; however, at 3 days the growth curve for the RANsiRNA treatment changed in a positive way to a slower rate as shown in figure 8.

Example 19. *In vivo* use of chemically modified polycation for RAF-1-siRNA delivery.

The experimental protocol described in Example 20 is repeated but substituting SCID-mice, 5 million HuH7 liver tumor cells to generate tumors, and RAF-1-siRNA in the therapy group. RAF-1-siRNA- a small interfering RNA directed against RAF-1, an important biological molecule for many cancer cells.

Example 20. *In vivo* use of chemically modified polycation for PLK-1 –SiRNA delivery.

The experimental protocol described in Example 19 is repeated, but substituting tumor cells that are known to express PLK-1 and have been demonstrated to exhibit cell death after transfection with PLK-1 siRNA in cell culture. PLK-1-siRNA - a small interfering RNA directed against polo-like kinase 1, an important regulator of cell cycle progression. Knockdown of expression leads to toxic effects

Example 21. Formation and characterization of polyplexes from Example 19.

Characterization of polyplex size is performed using a Malvern Zetasizer Nano ZS. This instrument is capable of measuring Zeta potential by conventional means and particle size by the technique of Quasielastic Laser Light Scattering. OEI-HD, 1.1 micrograms and OEI-PEG-transferrin, 0.12 micrograms are mixed in a total volume of 25 microliters of HBG (HEPES Buffered Glucose, a solution of 5 % glucose (weight/volume) containing 20 mM

HEPES at a pH of 7.3). The respective amount of siRNA (2.0 micorgrams) was diluted in another vial using HBG to 25 microliters. Subsequently the polymer solution was added to the siRNA solution and mixing was performed by inverting the container 10 times. The resulting polyplex suspension had a particle size between 200 – 300 nm and a Zeta potential of –1.3 milivolts. Zeta Potential is the electrical potential associated with a colloidal particle moving in an electric field at the surface of shear between the particles stationary ion layer and the mobile ion diffusion layer.

Example 22. Coupling of activated bifunctional PEG to the nucleic acid carrier OEI-HD-1 followed by coupling of transferrin to the pendant activated PEG endgroups

OEI (34K), 300 mg (~$9x10^{-6}$ mole) were dissolved in 5 ml of 100 mM HEPES buffer, pH 8.6. In a separate vial, 75 mg (66% purity by NMR, ~$1x10^{-5}$ mole) OPSS-PEG(5K)-SPA (ortho-pyridyl disulfide-polyethylene glycol-succinimidyl propionate) were dissolved in 4 ml of ethanol. Both solutions were combined and stirred for 2 hours at room temperature. The reaction solution was subsequently subjected to ion exchange chromatography and the fraction containing the OPSS-PEG-OEI was collected. The volume of the purified product was reduced to 1 ml using a Centricon concentrator. The product was desalted using a PD10 column (pre-saturated w/ OEI). This was performed by adding 1 ml of the sample solution to the column followed by addition of 1.5 ml of water. The initial flow-through was discarded and the sample eluted in 2 ml of water. This solution was freeze-dried and analyzed by proton NMR, which indicated one pyridyldithio group per 714 OEI repeat units. This intermediate was named OEI-HD-1-polyethylene glycol-2-pyridyldithio-propionamide or "OEI-PEG-OPSS" for short.

Reduction of OEI-PEG-OPSS to OEI-PEG-SH

25 mg of the freeze-dried OEI-PEG-OPSS were dissolved in 1.5 of 100 mM HEPES buffer. Subsequently, 40 mg (~$2.6x10^{-4}$ mole) of DL-dithiothreitol (DTT) were added into the sample solution and it was stirred for 30 minutes. To monitor the progress of the reaction, 10 µl of the sample solution were diluted to 500 µl with 100 mM HEPES buffer and an absorption scan was performed using an UV/VIS photometer. An observation of a local maximum @ 343 nm confirmed the progress of this reaction. Using the PD10 column, purified the reaction solution. This was accomplished by adding 1.5 ml of the reaction solution to the column (pre-saturated w/ OEI and equilibrated with HBS Buffer) followed by

addition of 1 ml of HBS buffer (HBS buffer was bubbled w/ argon gas before use). The initial flow-through was discarded and the sample eluted in 2 ml of HBS. The concentration of the OEI-PEG-SH sample solution was 5 mg/ml as determined by a copper complexation assay. For storage, the sample solution was bubbled with argon for five minutes before sealing.

Modification of Transferrin with N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP)

Transferrin (60 mg) were dissolved in 3 ml of HBS buffer. Subsequently, 3.5 mg of SPDP were dissolved in 7 ml of ethanol (carefully warm the solution for complete dissolution). Immediately 0.5 ml of the SPDP solution were pipetted into the transferrin solution and stirred gently for one hour.

The reaction solution was transferred to a YM-10 Centricon concentrator to reduce the volume to 1 ml. Four more 1 ml buffer exchanges were done on the Centricon concentrator. The purified transferrin-SPDP solution was bubbled with argon gas for five minutes.

Coupling of OEI-PEG-SH with Transferrin-SPDP

10 mg of the OEI-PEG-SH dissolved in 2 ml of HBS buffer solution and 2.7 ml of the purified and activated transferrin solution were united in a new scintillation vial and stirred gently overnight at room temperature. Then the unreacted sulfhydryl groups were quenched with 5 mg ($4x10^{-5}$ mole) N-ethyl-maleimide and stirred for 30 minutes.

OEI-PEG –transferrin was purified by ion exchange chromatography. The volume reduced using a Centricon concentrator, and the product desalted as previously described.

The concentration of the polyamine (OEI) in the bioconjugate was 1.3 mg/ml determined by the copper complexation assay and the transferrin assayed at 2.9 mg/ml as determined by UV spectroscopy.

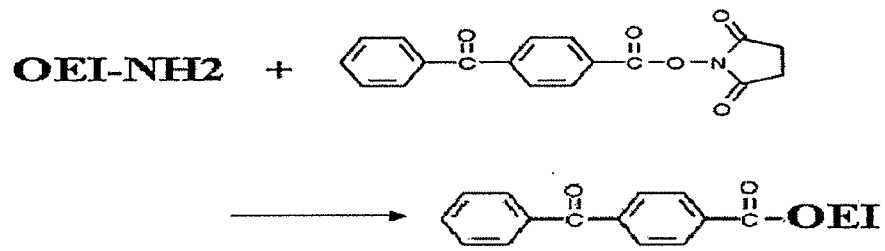
Incorporation of Iron into Transferrin

Iron loading into transferring is done by adding 1.25 µl of the Iron Loading Buffer per milligram of transferrin content into the sample, as described in Kursa M, Walker GF, Roessler V, Ogris M, Roedl W, Kircheis R, Wagner E., *Bioconjugate Chem.* 2003

The use of a shift assay to determine the binding affinity of siRNA to OEI-PEG 5K-transferrin.

A siRNA solution of 0.01 mg/ml was prepared from a non-specific siRNA stock solution. Transferred 20μl of the siRNA solution into a number of small plastic vials, followed by increasing amounts of OEI-PEG 5K-transferrin in HBG buffer solution, keeping the total volume in each vial constant at 40 ul. The weight ratio of OEI-PEG 5K-transferrin-to- siRNA ranged from 0 to 10. After pipetting, the vial contents were mixed well and let stand for 10 minutes. Then 20 μl aliquots from each vial were transferred to their corresponding well in an agarose gel plate. The agarose gel plate was placed in an electrophoresis apparatus set at 75 volts. After 15 minutes the plate was removed and photodocumented. We found that there was no migration band of siRNA observed in the wells of weight ratio 1.5 or higher. This indicates that all siRNA was complexed with OEI(34K)-PEG(5K)-transferrin at weight ratios of 1.5 and higher.

Example 23: Coupling of benzoylbenzoic acid to Compounds of Formula I or Ia



(RLU=relative luminescence units; C:P+ carrier to plasmid or siRNA ration w/w; luc= anti-luciferase siRNA; scr= scrambler siRNA control; and lip = lipofectamine transfection agent.)

Benzoiylbenzoic acid-succinimidyl ester (Invitrogen # 1577) was used and the reaction was perfomed in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3,). 20 mg polymer (described below) were dissolved in 2 ml HBS and pH was adjusted to be 7.3. Subsequently different amounts of benzoylbenzoic acid-NHS ester were added in 1 ml anhydrous DMSO.

All reactions were run for 12 hours before dilution to approximately 6 ml was performed using deionised water. Subsequently dialysis was performed and after that conjugates were freeze-dried.

TABLE 1

Dialysis membranes used:

Basal Polymer	Membrane MWCO
PEI 2kDa	1 kDa
OEI 5kDa	3 kDa
OEI 30kDa	10 kDa

TABLE 2

Actual degrees of coupling for the conjugates synthesized

Conjugate	Benzoylbenzoic acid per polymer (NMR)
PEI 2kDa low	2.5
PEI 2kDa medium	7
PEI 2kDa high	15
OEI 5k low	1.8
OEI 5k medium	12
OEI 5k high	20
OEI 30k low	1
OEI 30k medium	3
OEI 30k high	8

The samples were freeze-dried again and dissolved in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3). From these solutions concentrations were determined against unmodified polymer using the copper assay.

Size determinations via Dynamic Laser Light Scattering

0.5 microg of siRNA were complexed with the respective amount of OEI in a total volume of 50 microliters.

TABLE 3

C:P	PEI 2K benzo-1 low	PEI 2K benzo-2 medium	PEI 2K benzo-3 high
0.3	254	370	392
0.6	202	239	204
1.2	178	198	194
2.0	182	195	189

TABLE 4

C:P	OEI 5K benzo-1	OEI 5K benzo-2	OEI 5K benzo-3
0.3	370	72	mult
0.6	303	350	195
1.2	196	210	260
2.0	190	121	99

mult= multiple peaks

TABLE 5

C:P	OEI 30K benzo-1	OEI 30K benzo-2	OEI 30K benzo-3
0.3	436	457	342
0.6	151	mult	249
1.2	mult	mult	116
2.0	mult	mult	120

TABLE 6
Knockdown efficiency H1299 cells, 0.5 microg siRNA per well

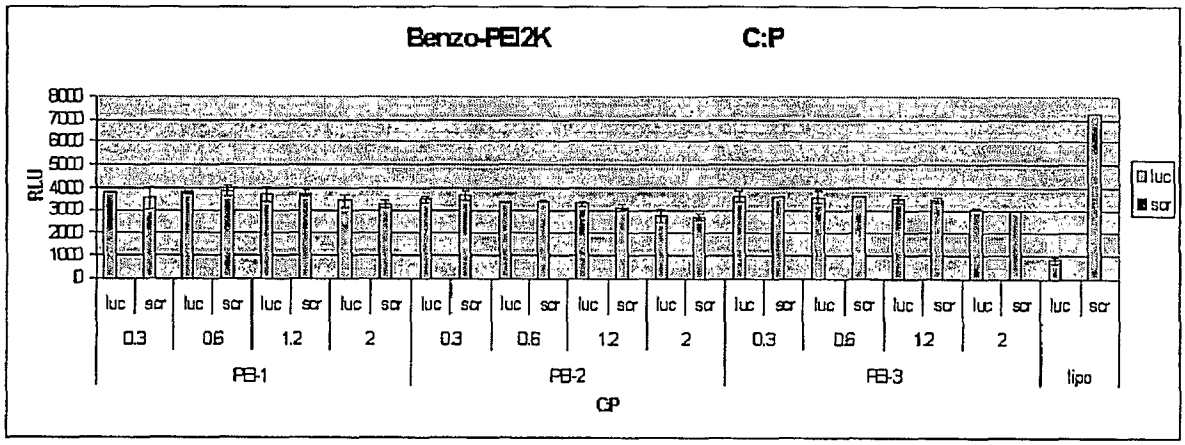


TABLE 7

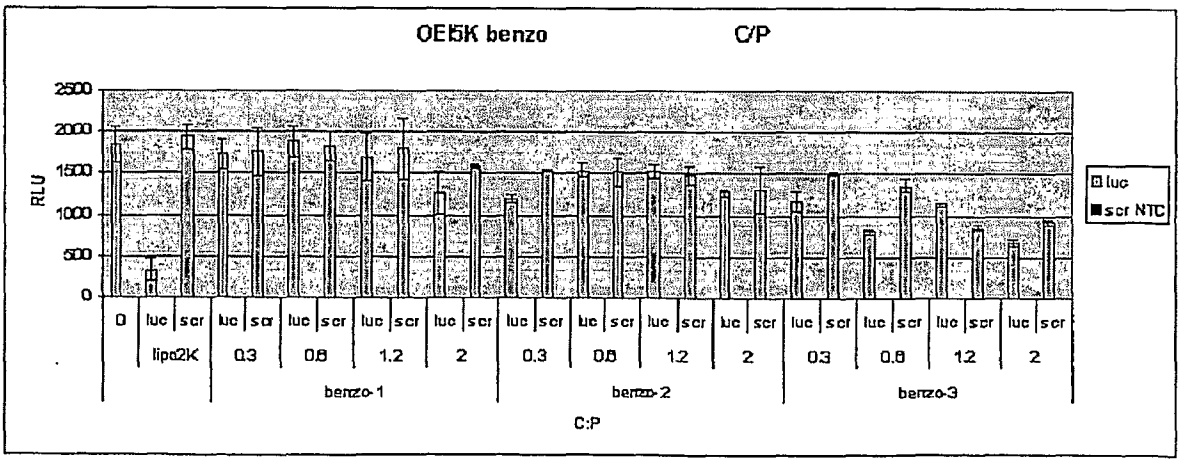


TABLE 8

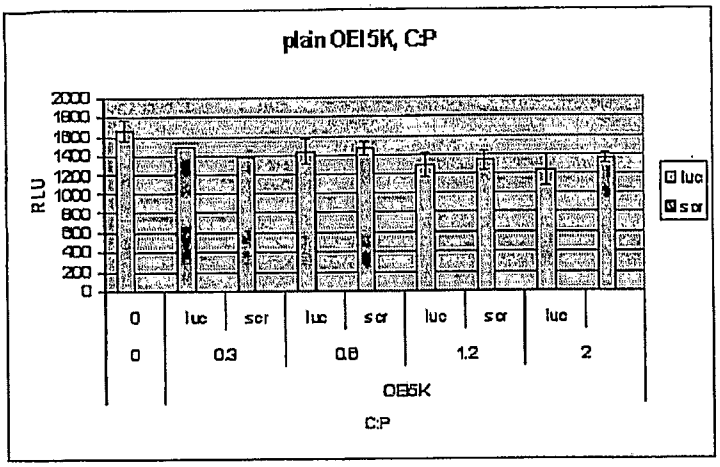


TABLE 9

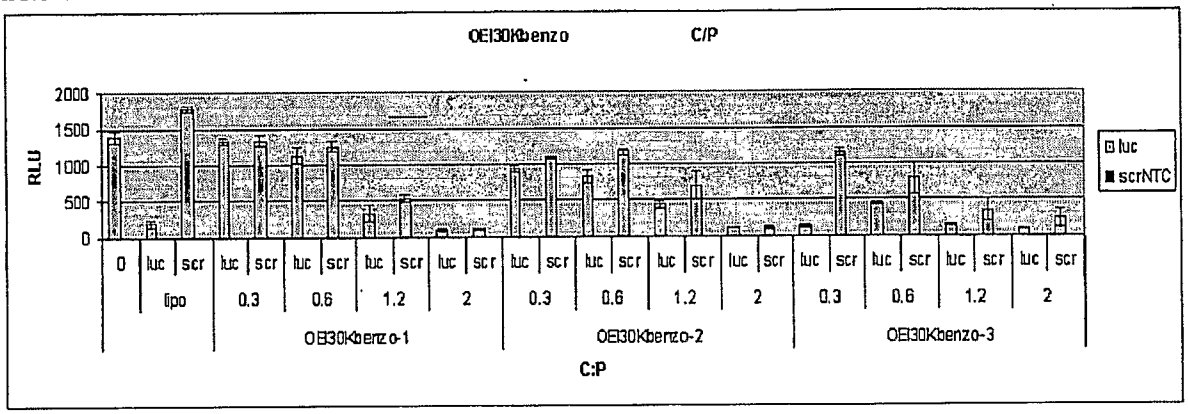
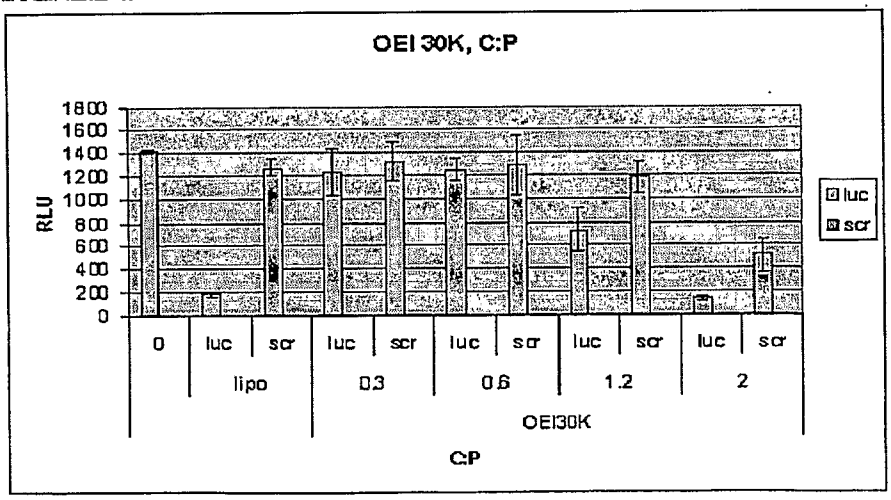


TABLE 10



Example 24: Coupling of lauric acid to Compounds of Formula I or Ia

Lauric acid N-hydroxy-succinimidyl ester (Sigma-Aldrich # OL3900-5g, Lot # 087H5174) was used and the reaction was perfomed in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3).

20 mg polymer were dissolved in 2 ml HBS and pH was adjusted to be 7.3. Subsequently different amounts of lauric acid NHS ester were added in 1 ml anhydrous DMSO.

TABLE 11

PEI 800 Da and PEI 2 kDa

PEI 800 Da	PEI 2 kDa	mg lauric acid NHS	acid per PEI	Remarks
20 mg	-	7.4	1	none
20 mg	-	29.7	4	precipitation
20 mg	-	59.4	8	precipitation
-	20 mg	5.9	2	none
-	20 mg	23.8	8	precipitation
-	20 mg	35.6	12	precipitation

TABLE 12

OEI 5 kDa, 9 kDa and 30 kDa

20 mg	mg lauric acid NHS	acid per PEI	Remarks
5kDa	2.4	2	None
5kDa	11.9	10	None
5kDa	29.7	25	cloudiness
9kDa	2.7	4	None
9kDa	10	15	None
9kDa	27	40	precipitation
30kDa	1.2	6	None
30kDa	5	25	None
30kDa	20	100	precipitation

All reactions were run for 12 hours before dilution to approximately 6 ml was performed using deionised water. Subsequently dialysis was performed and after that conjugates were freeze-dried.

TABLE 13

Conjugate basal polymer	Dialysis Membrane cut off
PEI 800	500
PEI 2 kDa	1 kDa
OEI 5 kDa	3 kDa
OEI 9 kDa	3 kDa
OEI 30 kDa	10 kDa

Subsequently the conjugates were dissolved in D2O and submitted for ^{1}H-NMR.

The following actual coupling degrees were determined via NMR.

Coupling degrees based on NMR calculations:

TABLE 14

Conjugate	lauric acid per polymer
PEI 800	could not be processed due to cloudiness
PEI 2k	0.5
OEI 5k low	1.6
OEI 5k medium	8
OEI 5k high	could not be processed due to low solubility
OEI 9k low	4
OEI 9k medium	12
OEI 30k low	4
OEI 30k medium	22

The samples were freeze-dried again and dissolved in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3).

From these solutions concentrations were determined against unmodified polymer using the copper assay.

Size determinations via Dynamic Laser Light Scattering

0.5 microg of siRNA were complexed with the respective amount of OEI in a total volume of 50 microliters. Experiments were carried out in HBS.

TABLE 15

size			
C:P	PEI 800	PEI2K	PEI2K-LA
0.3	297	116	136
0.6	250	mult	113
1.2	215	mult	121
2.0	192	mult	123

TABLE 16

size			
C:P	OEI5K	OEI5K LA1	OEI5K LA2
0.3	mult	83	76
0.6	mult	mult	72
1.2	mult	mult	mult
2.0	mult	mult	mult

TABLE 17

size		
C:P	OEI 9K LA-1	OEI 9K LA-2
0.3	162	88
0.6	124	80
1.2	mult	88
2.0	mult	mult

Plain OEI 9k gave multiple peaks over the whole range !

TABLE 18

size		
C:P	OEI 30K LA-1	OEI 30K LA-2
0.3	108	94
0.6	mult	mult
1.2	mult	mult
2.0	mult	mult

Knockdown efficiency H1299 cells, 0.5 microg siRNA per well.
PEI 800 and PEI2k and derivatives thereof

TABLE 19

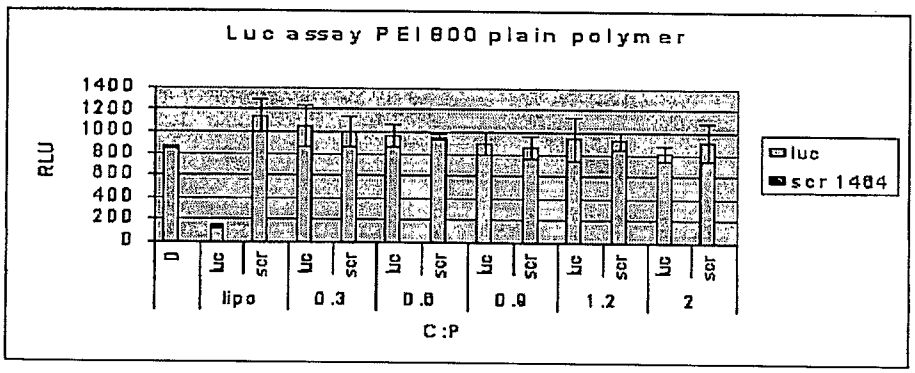


TABLE 20

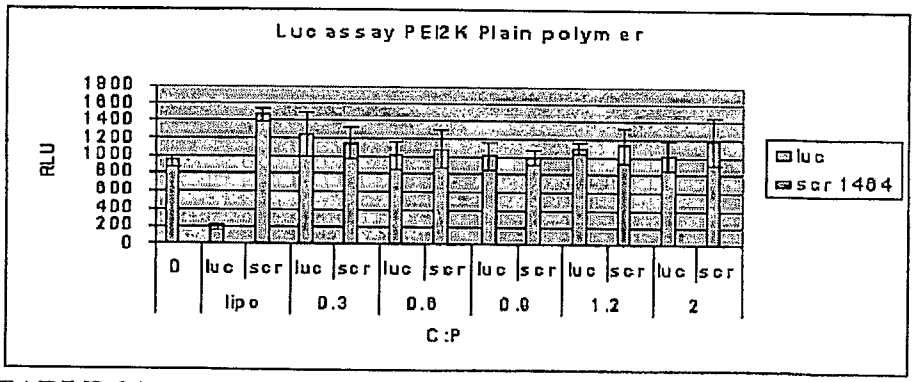
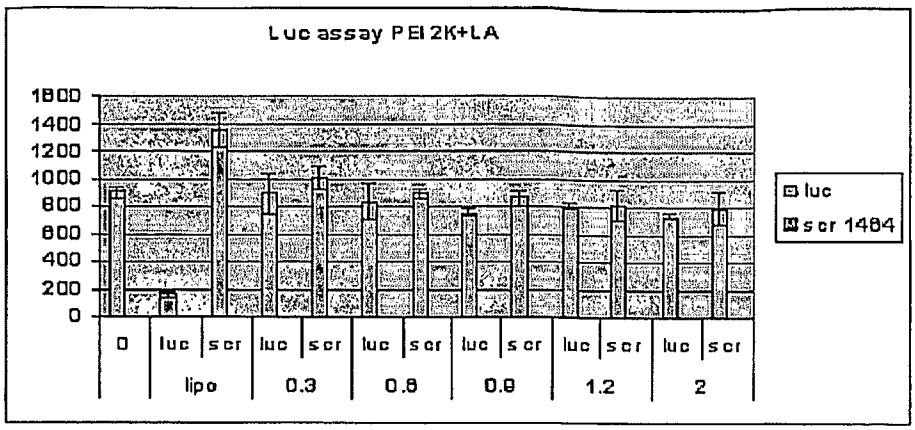


TABLE 21



OEI 5k and derivatives thereof

TABLE 22

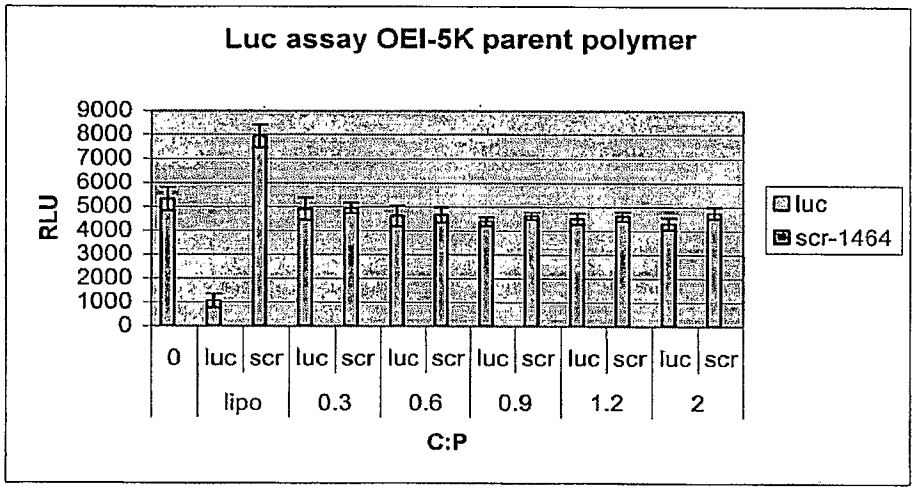


TABLE 23

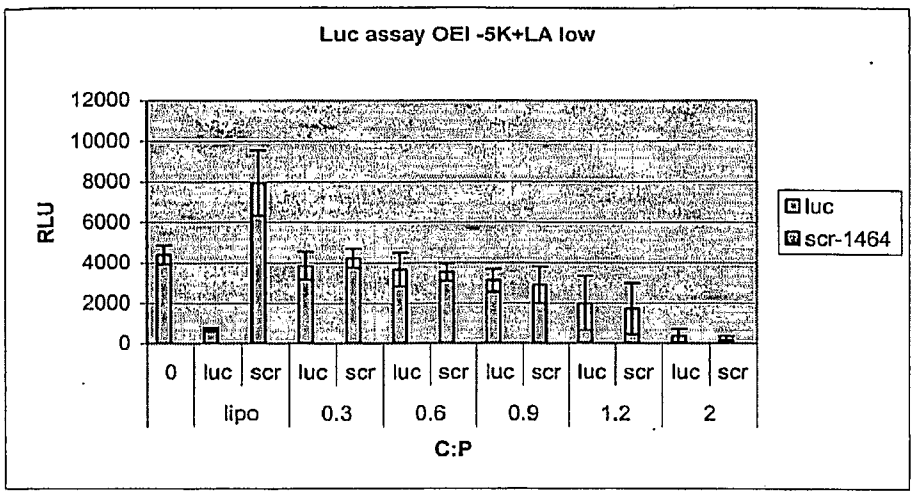
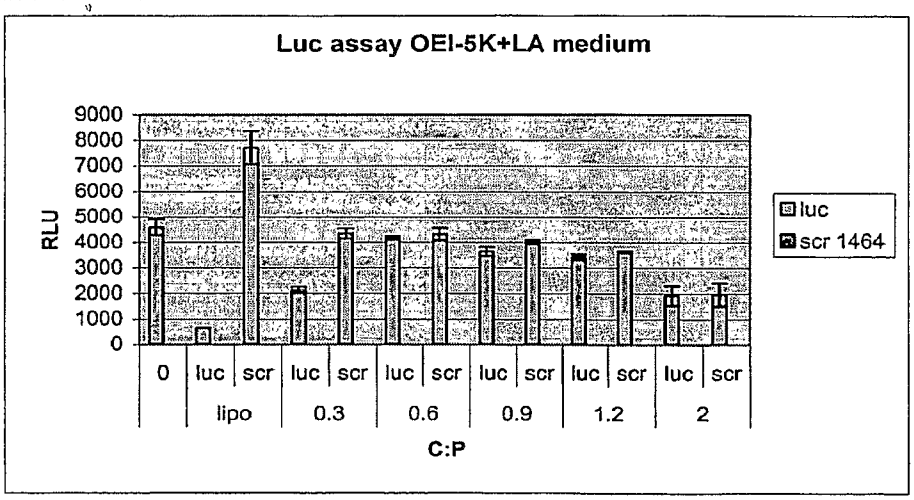
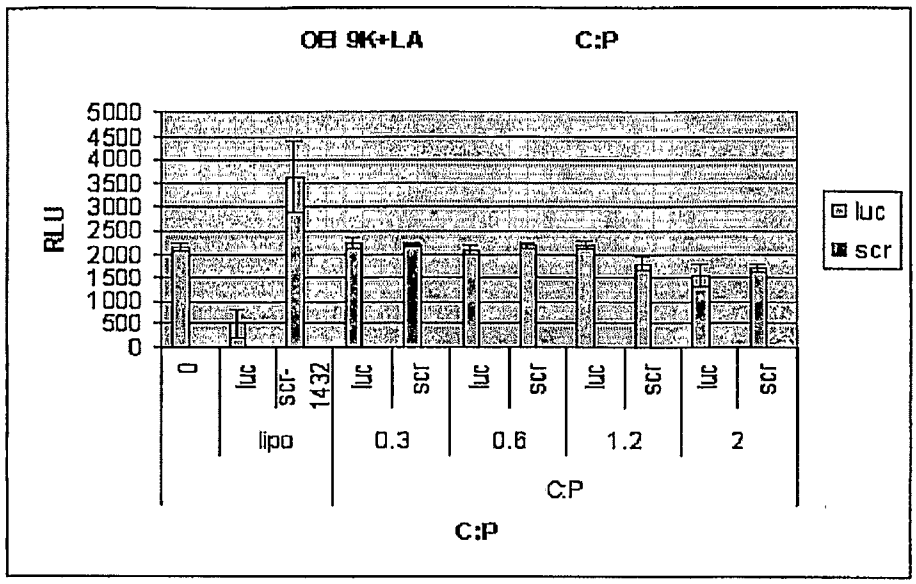


TABLE 24



OEI 9k derivatives

TABLE 24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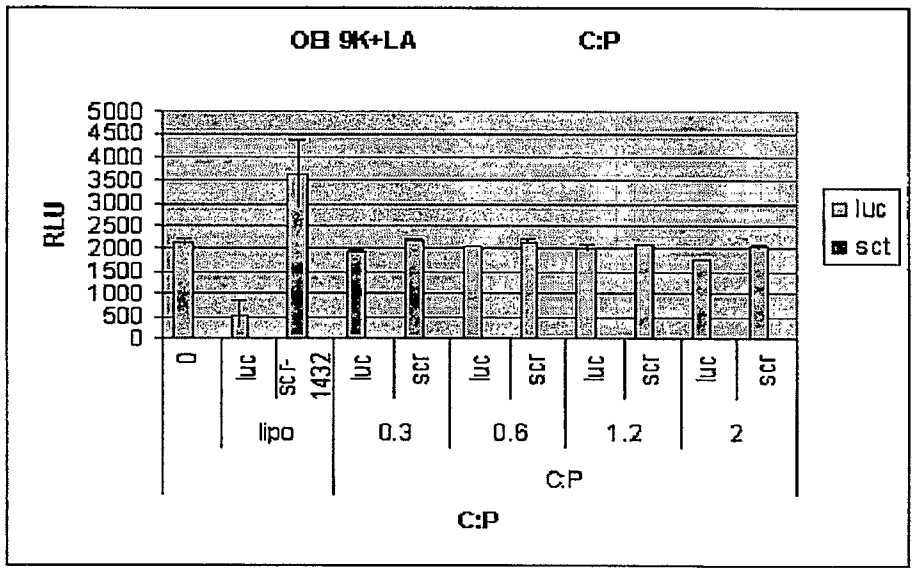
C:P
RLU
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
luc
scr
0
luc
scr-1432
luc
scr
luc
scr
luc
scr
luc
scr
lipo
0.3
0.6
1.2
2
C:P
C:P

TABLE 25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C:P
RLU
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
luc
sct
0
luc
scr-1432
luc
scr
luc
scr
luc
scr
luc
scr
lipo
0.3
0.6
1.2
2
C:P
C:P

OEI 30 k derivatives

TABLE 26

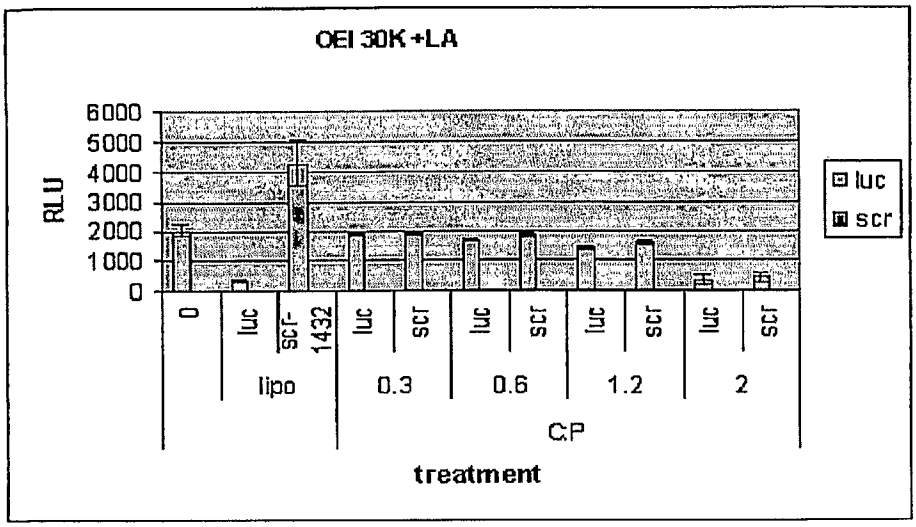
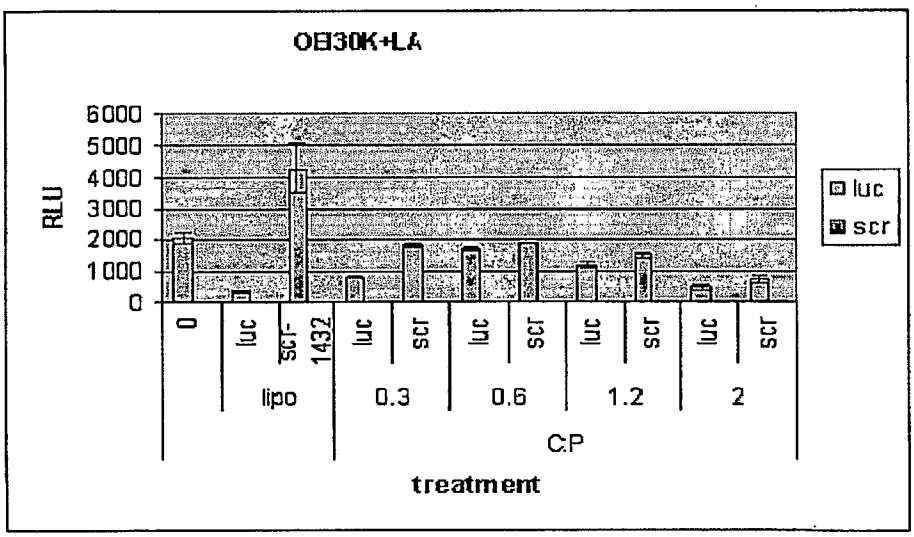


TABLE 27



Example 23: Coupling of lauric acid to PEIs/OEIs and physicochemical as well as biological evaluation of conjugates

Lauric acid N-hydroxy-succinimidyl ester (Sigma-Aldrich # OL3900-5g, Lot # 087H5174) was used and the reaction was perfomed in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3).

20 mg polymer were dissolved in 2 ml HBS and pH was adjusted to be 7.3. Subsequently different amounts of lauric acid NHS ester were added in 1 ml anhydrous DMSO.

PEI 800 Da and PEI 2 kDa

TABLE 28

PEI 800 Da	PEI 2 kDa	mg lauric acid NHS	acid per PEI	Remarks
20 mg	-	7.4	1	none
20 mg	-	29.7	4	precipitation
20 mg	-	59.4	8	precipitation
-	20 mg	5.9	2	none
-	20 mg	23.8	8	precipitation
-	20 mg	35.6	12	precipitation

OEI 5 kDa, 9 kDa and 30 kDa

TABLE 29

20 mg	mg lauric acid NHS	acid per PEI	Remarks
5kDa	2.4	2	None
5kDa	11.9	10	None
5kDa	29.7	25	cloudiness
9kDa	2.7	4	None
9kDa	10	15	None
9kDa	27	40	precipitation
30kDa	1.2	6	None
30kDa	5	25	None
30kDa	20	100	precipitation

All reactions were run for 12 hours before dilution to approximately 6 ml was performed using deionised water. Subsequently dialysis was performed and after that conjugates were freeze-dried.

TABLE 30

Conjugate basal polymer	Dialysis Membrane cut off
PEI 800	500
PEI 2 kDa	1 kDa
OEI 5 kDa	3 kDa
OEI 9 kDa	3 kDa
OEI 30 kDa	10 kDa

Subsequently the conjugates were dissolved in D2O and submitted for ^{1}H-NMR. The following actual coupling degrees were determined via NMR.

Coupling degrees based on NMR calculations:

TABLE 31

Conjugate	lauric acid per polymer
PEI 800	could not be processed due to cloudiness
PEI 2k	0.5
OEI 5k low	1.6
OEI 5k medium	8
OEI 5k high	could not be processed due to low solubility
OEI 9k low	4
OEI 9k medium	12
OEI 30k low	4
OEI 30k medium	22

The samples were freeze-dried again and dissolved in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3.

From these solutions concentrations were determined against unmodified polymer using the copper assay.

Size determinations via Dynamic Laser Light Scattering

0.5 microg of siRNA were complexed with the respective amount of OEI in a total volume of 50 microliters. Experiments were carried out in HBS.

TABLE 32

size			
C:P	PEI 800	PEI2K	PEI2K-LA
0.3	297	116	136
0.6	250	mult	113
1.2	215	mult	121
2.0	192	mult	123

TABLE 33

size			
C:P	OEI5K	OEI5K LA1	OEI5K LA2
0.3	mult	83	76
0.6	mult	mult	72
1.2	mult	mult	mult
2.0	mult	mult	mult

TABLE 34

size		
C:P	OEI 9K LA-1	OEI 9K LA-2
0.3	162	88
0.6	124	80
1.2	mult	88
2.0	mult	mult

Plain OEI 9k gave multiple peaks over the whole range !

TABLE 35

size		
C:P	OEI 30K LA-1	OEI 30K LA-2
0.3	108	94
0.6	mult	mult
1.2	mult	mult
2.0	mult	mult

Plain OEI 30k gave multiple peaks over the whole range !

Knockdown efficiency H1299 cells, 0.5 microg siRNA per.

PEI 800 and PEI2k and derivatives thereof

TABLE 36

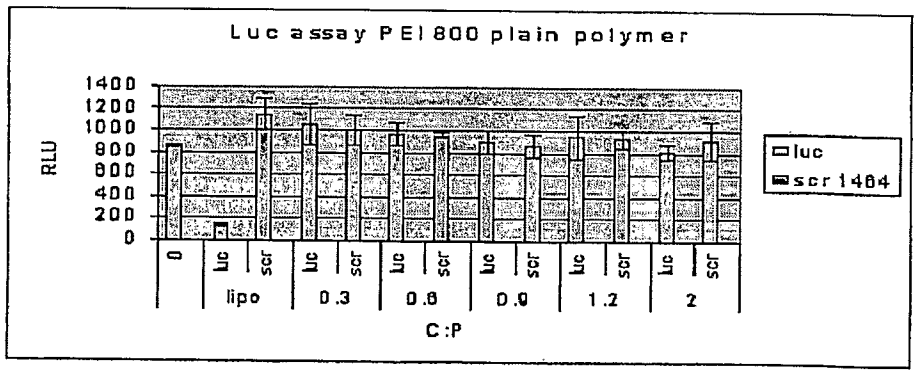


TABLE 37

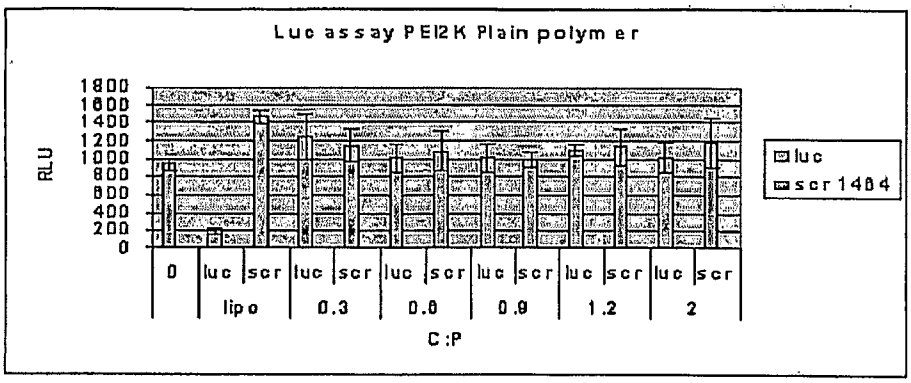
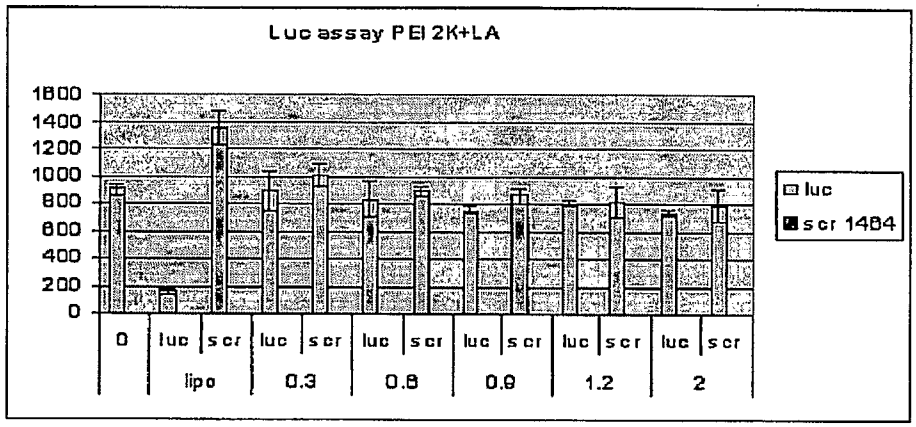


TABLE 38



<u>OEI 5k and derivatives thereof</u>

TABLE 39

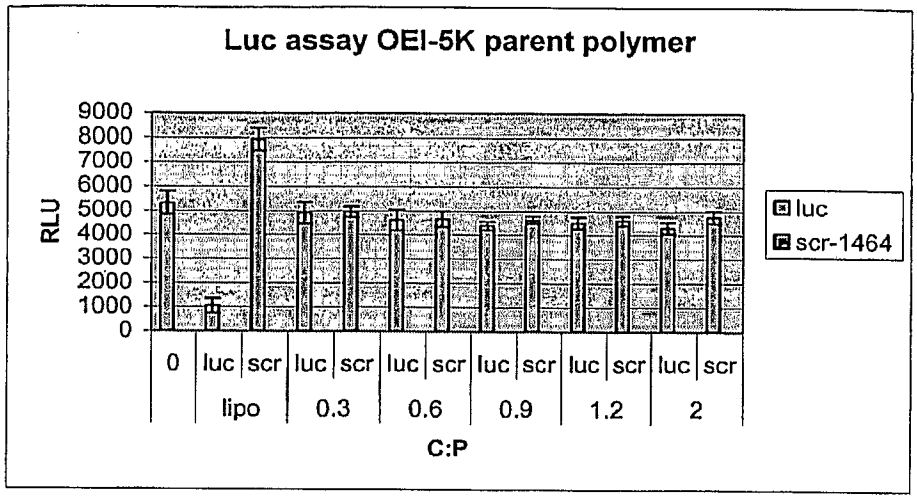


TABLE 40

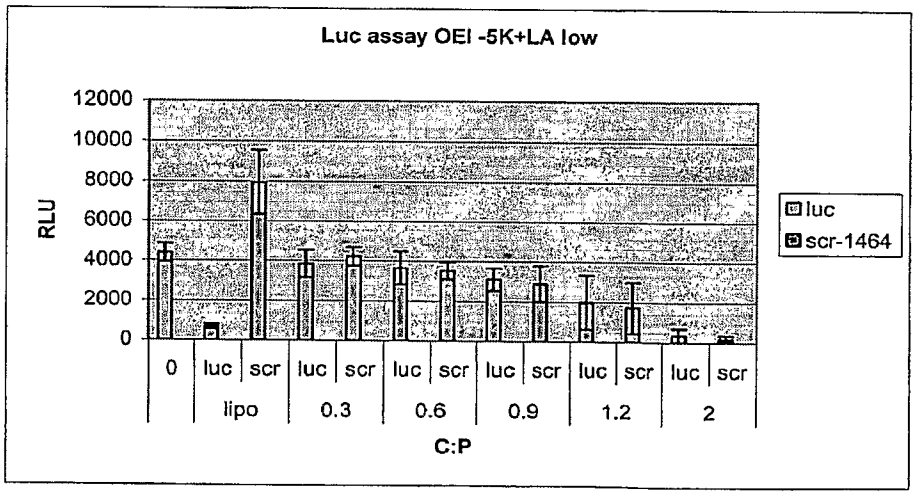
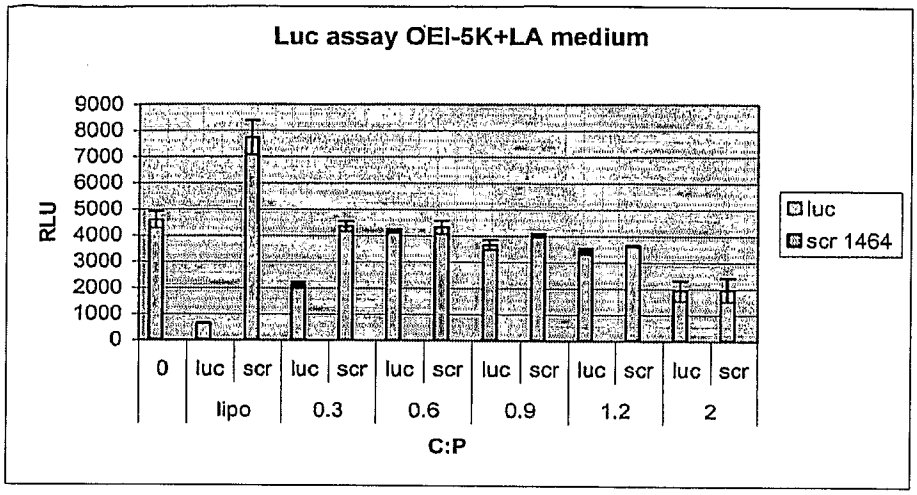


TABLE 41



OEI 9k derivatives

TABLE 42

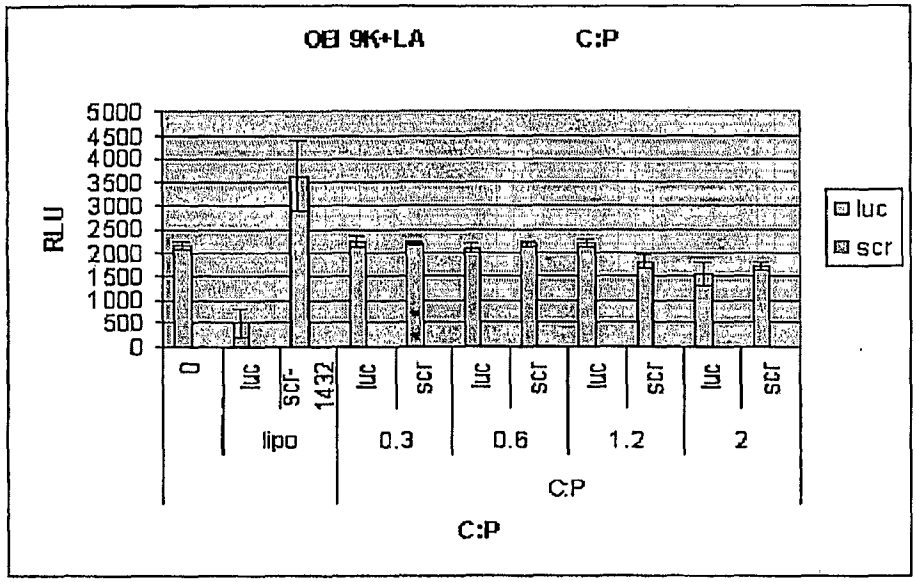
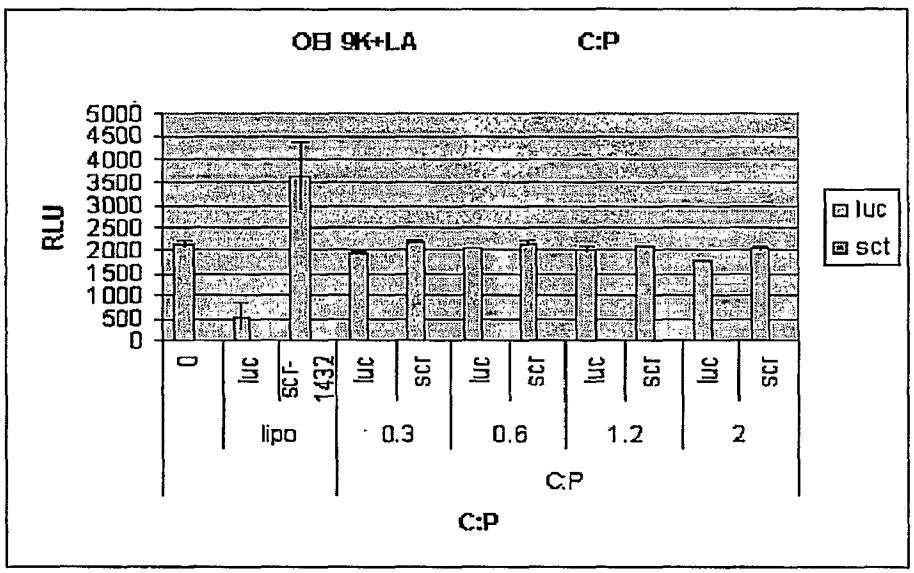

OB 9K+LA
C:P
RLU
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
luc
scr
0
luc
scr-1432
luc
scr
luc
scr
luc
scr
luc
scr
lipo
0.3
0.6
1.2
2
C:P
C:P

TABLE 43


OB 9K+LA
C:P
RLU
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
luc
sct
0
luc
scr-1432
luc
scr
luc
scr
luc
scr
luc
scr
lipo
0.3
0.6
1.2
2
C:P
C:P

OEI 30 k derivatives

TABLE 44

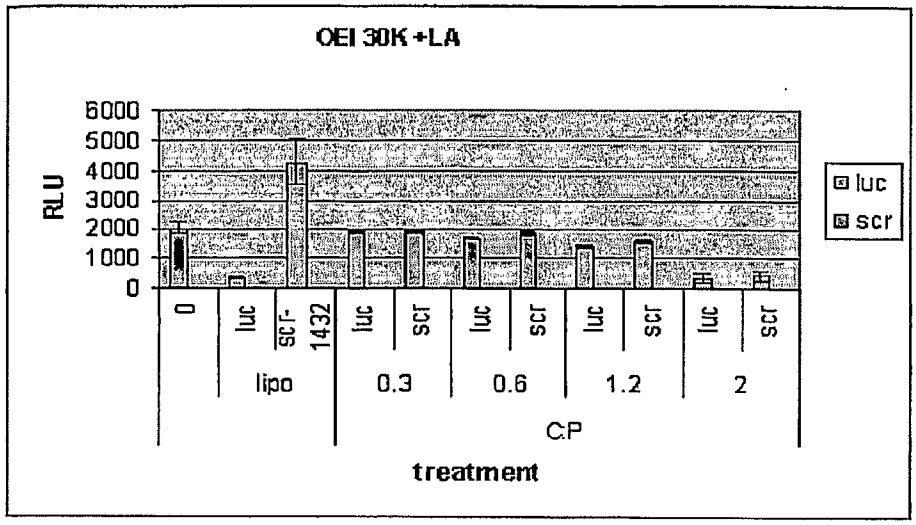
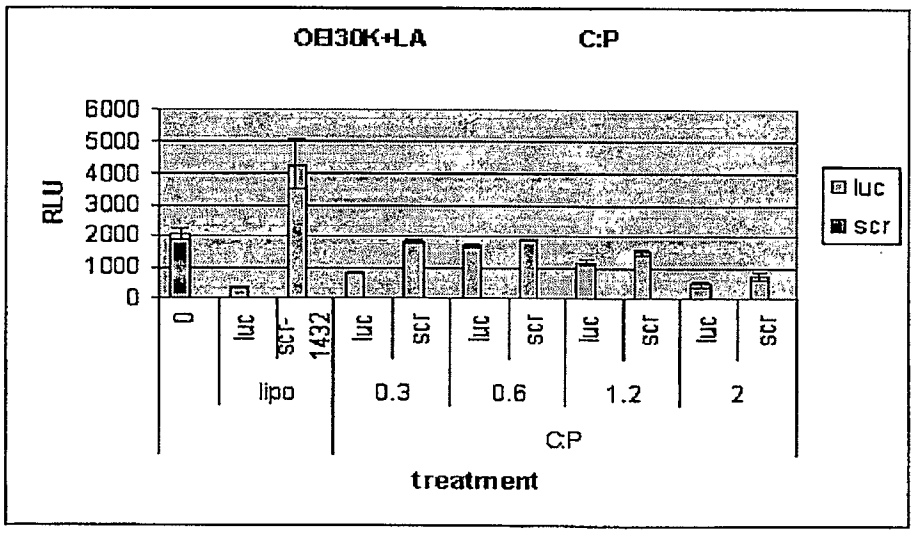
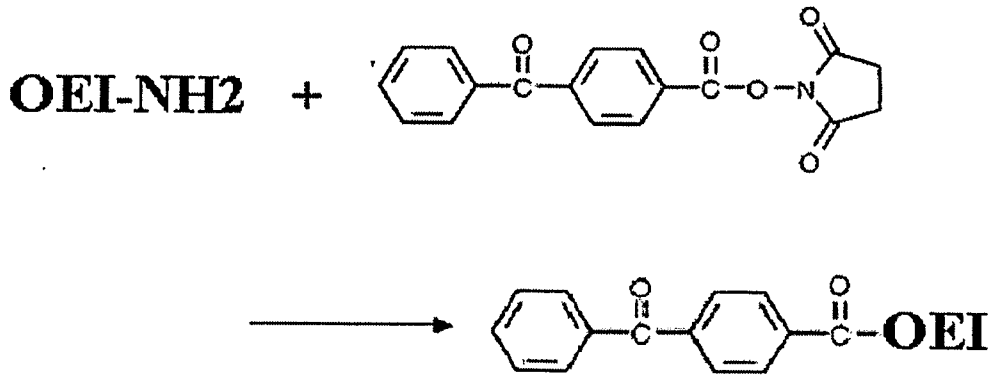


TABLE 45



Example 24: Coupling of benzoylbenzoic acid to PEIs/OEIs and physicochemical as well as biological evaluation of conjugates



Benzoiylbenzoic acid-succinimidyl ester (Invitrogen # 1577) was used and the reaction was perfomed in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3).

20 mg polymer were dissolved in 2 ml HBS and pH was adjusted to be 7.3. Subsequently different amounts of benzoylbenzoic acid-NHS ester were added in 1 ml anhydrous DMSO. All reactions were run for 12 hours before dilution to approximately 6 ml was performed using deionised water. Subsequently dialysis was performed and after that conjugates were freeze-dried.

TABLE 46

20 mg polymer	benzoylbenzoic acid NHS ester [mg]	acid per polymer
PEI 2 kDa	3.2	1
PEI 2 kDa	10	3
PEI 2 kDa	19	6
OEI 5 kDa	2.5	2
OEI 5 kDa	12.9	10
OEI 5 kDa	26	20
OEI 30 kDa	1.3	6
OEI 30 kDa	4.3	20
OEI 30 kDa	10.8	50

Dialysis membranes used:

TABLE 47

Basal Polymer	Membrane MWCO
PEI 2kDa	1 kDa
OEI 5kDa	3 kDa
OEI 30kDa	10 kDa

Actual degrees of coupling for the conjugates synthesized

TABLE 48

Conjugate	Benzoylbenzoic acid per polymer (NMR)
PEI 2kDa low	2.5
PEI 2kDa medium	7
PEI 2kDa high	15
OEI 5k low	1.8
OEI 5k medium	12
OEI 5k high	20
OEI 30k low	1
OEI 30k medium	3
OEI 30k high	8

The samples were freeze-dried again and dissolved in HBS (Hepes buffered Saline, 10 mM HEPES, 150 mM NaCl, pH 7.3).

From these solutions concentrations were determined against unmodified polymer using the copper assay.

Size determinations via Dynamic Laser Light Scattering

0.5 microg of siRNA were complexed with the respective amount of OEI in a total volume of 50 microliters.

TABLE 49

C:P	PEI 2K benzo-1 low	PEI 2K benzo-2 medium	PEI 2K benzo-3 high
0.3	254	370	392
0.6	202	239	204
1.2	178	198	194
2.0	182	195	189

TABLE 50

C:P	OEI 5K benzo-1	OEI 5K benzo-2	OEI 5K benzo-3
0.3	370	72	mult
0.6	303	350	195
1.2	196	210	260
2.0	190	121	99

mult= multiple peaks

TABLE 51

C:P	OEI 30K benzo-1	OEI 30K benzo-2	OEI 30K benzo-3
0.3	436	457	342
0.6	151	mult	249
1.2	mult	mult	116
2.0	mult	mult	120

Knockdown efficiency H1299 cells, 0.5 microg siRNA per well.

TABLE 52

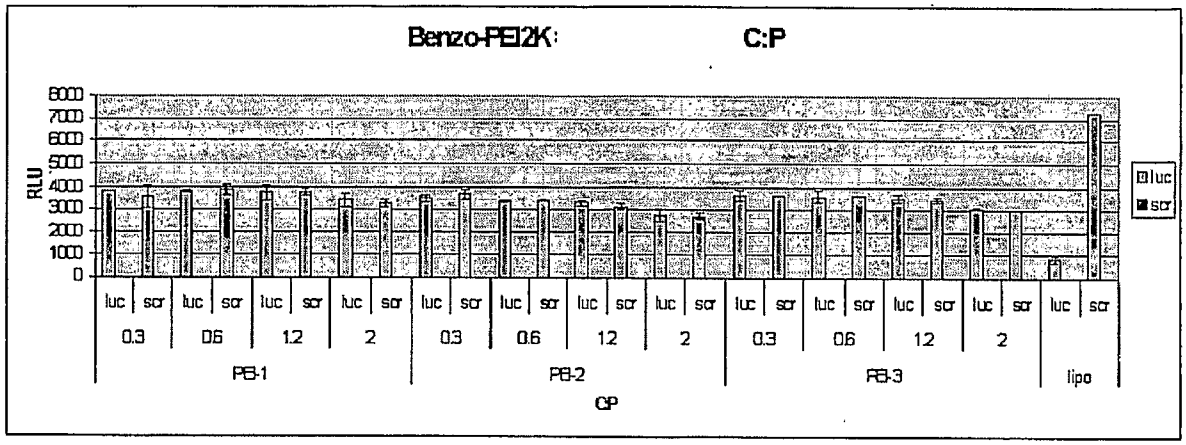


TABLE 53

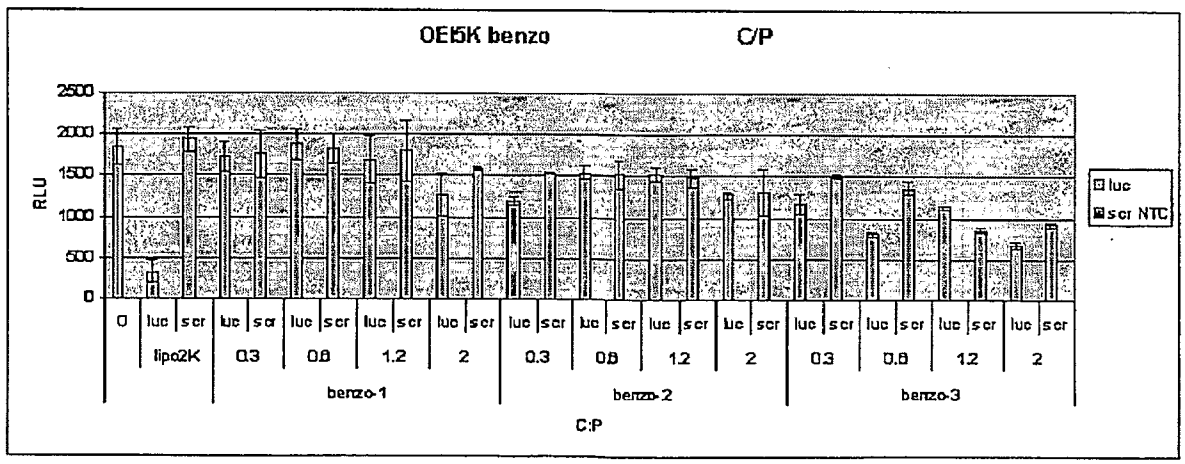


TABLE 54

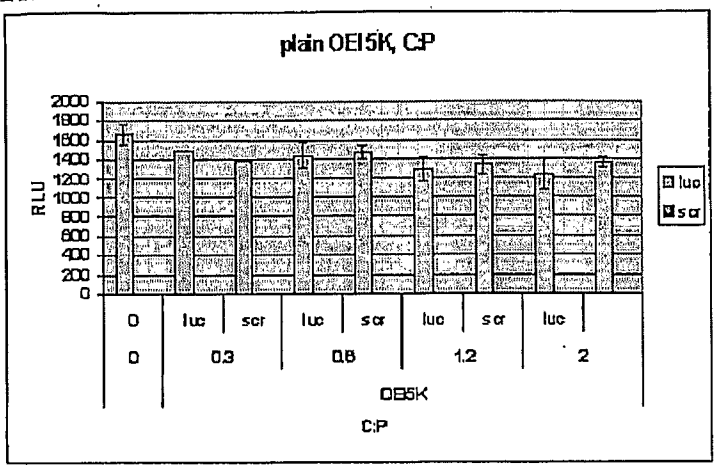


TABLE 55

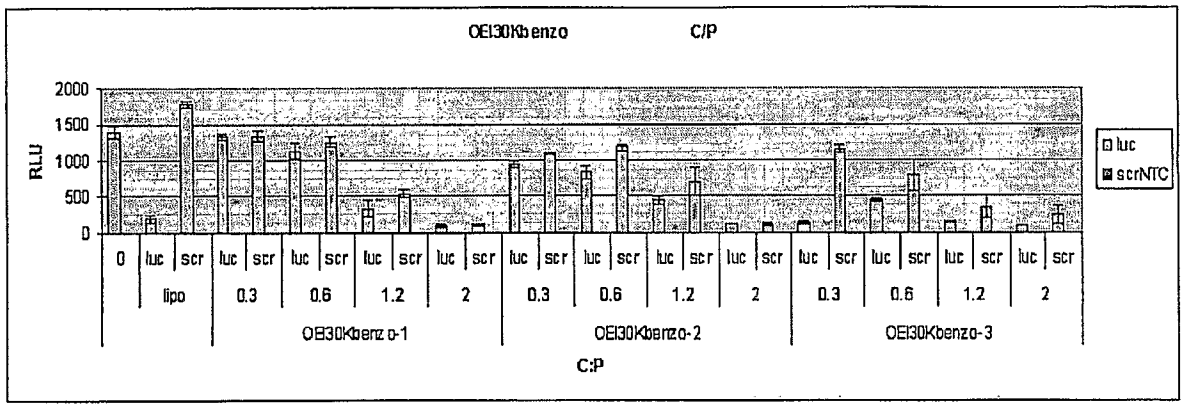
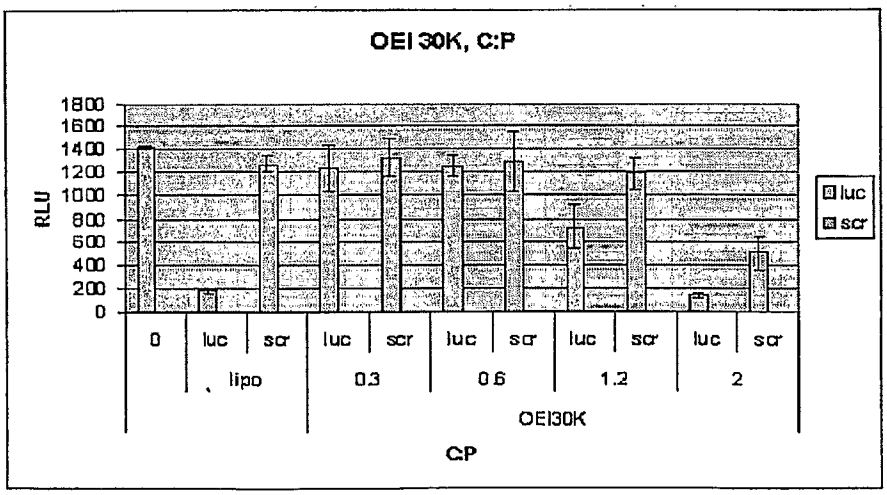


TABLE 56



WHAT IS CLAIMED IS

1. A polymer formed by polycations chemically linked by propionylamide units, wherein said polymer is useful as a non-viral carrier for nucleic acid delivery.
2. The polymer of claim 1 wherein the polycation is polyethyleneimine (PEI).
3. The polymer of claim 1 that is useful for siRNA delivery.
4. The polymer of claim 1 further comprising a shielding ligand.
5. The polymer of claim 4, wherein the shielding ligand is polyethylene glycol (PEG).
6. The polymer of claim 5 further comprising a targeting ligand.
7. The polymer of claim 6 wherein the targeting ligand is transferrin.
8. The polymer of claim 4 further comprising coupling with a polynucleotide.
9. The polymer of claim 5 further comprising coupling with a polynucleotide.
10. The polymer of claim 6 further comprising coupling with a polynucleotide.
11. The polymer of claim 7 further comprising coupling with a polynucleotide.
12. A method of making the polymer of claim 1, wherein the polymer is cross-linked by the Michael addition of a fraction of the polymer's amines to vinylic groups of cross-linking agent and further modified by N-acylation of pendant ester groups.
13. The method of claim 12 wherein the polymer is further modified by addition of free ester, anhydryde, or acylhalide.
14. The method of claim 12 wherein the cross-linking can occur at both the primary and secondary amines of the polymer structure.
15. The method of claim 12 wherein the cross-linking agents comprise ester monomers of the following agents: acrylate, methacrylate, ethylene glycol diacrylate, ethylene glycol dimethacrylate, 1,6 hexanediol diacrylate, and polyethylene glycol 600 diacrylate.
16. The method of claim 15 wherein the cross linking agent is 1,6. hexanediol diacrylate.
17. A method of delivering genes to target tissue *in vivo* using the polymer of claim 1.
18. A method of delivering siRNA to cells in culture using the polymer of claim 1.
19. A method of delivering siRNA to tissue *in vivo* using the polymer of claim 1.
20. The method of claim 19, wherein the delivery of siRNA is for therapeutic purposes or for target validation.

21. A method of using the polymer of claim 1, wherein the polymer forms a targetable complexing agent that can bind entities of opposite charge and delivery them to target tissue.
22. A method of delivering therapeutic entity of interest to a patient using the polymer of claim 1, wherein the polymer forms a ionic complex or covalent bond with the therapeutic entity of interest.
23. The method of claim 22, wherein the therapeutic entity of interest comprises cytotoxic agents, endosomal lytic agents, and hydrophilic polymers.
24. A compound of Formula I comprising:

$$\left[[\text{polycation}]_a - [L]_b - [\text{polycation}]_c \right]_d$$

wherein:

polycation is a polyethyleneimine unit;

L is a non-ester linker;

a is an integer in the range of about 1 to about 20;

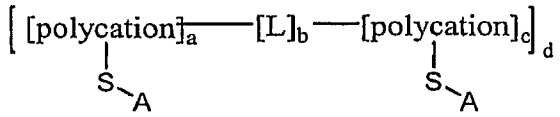
b is an integer in the range of about 1 to about 10;

c is an integer in the range of about 1 to about 20; and

d is an integer in the range of about 1 to about 1000.

25. The compound of claim 24 in which the polycation is oliogethyleneimine.
26. The compound of claim 24 in which L is selected from the group consisting of an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, and a succinamidyl linker moiety.
27. The non-ester linker of claim 26 in which L is a beta-aminopropionylamide linker moiety.
28. The compound of claim 24 further comprising a biomolecule.
29. The compound of claim 28 wherein said biomolecule is siRNA.
30. The compound of claim 24 having a weight average molecular weight in the range of about 800 Daltons to about 1,000,000 Daltons.
31. The compound of claim 24 having a weight average molecular weight in the range of about 20,000 Daltons to about 200,000 Daltons.
32. The compound of claim 24 having a weight average molecular weight in the range of about 20,000 Daltons to about 30,000 Daltons.

33. A compound of Formula I comprising:



wherein:

polycation is a polyethylenimine;

L a non-ester linker;

S is a spacer or is absent;

A is an agent or is absent;

a is an integer in the range of about 1 to about 20;

b is an integer in the range of about 1 to about 10;

c is an integer in the range of about 1 to about 20; and

d is an integer in the range of about 1 to about 1000.

34. The compound of claim 33 in which the polycation is oliogethyleneimine.

35. The compound of claim 33 in which L is selected from the group consisting of an amido linker moiety, an amino linker moiety, a carbonyl linker moiety, a carbamate linker moiety, a urea linker moiety, an ether linker moiety, a disulphide linker moiety, and a succinamidyl linker moiety.

36. The non-ester linker of claim 33 in which L is a beta-aminopropionylamide linker moiety.

37. The compound of claim 33 further comprising a biomolecule.

38. The compound of claim 37 wherein said biomolecule is siRNA.

39. The compound of claim 33 further comprising an agent and optionally S.

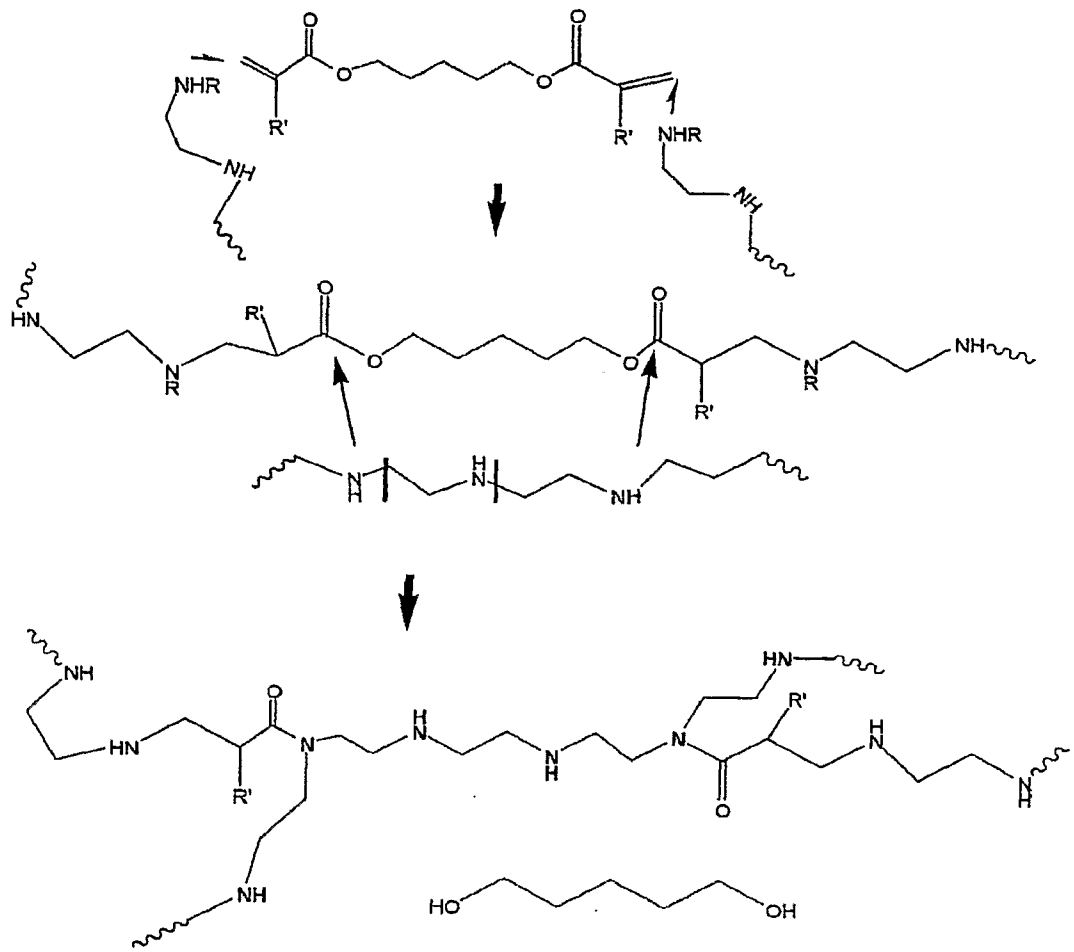
40. The compound of claims 38 or 39 wherein said agent is selected form the group consisting of an agent that facilitates receptor recognition, internalization, escape of the biomolecule from cell endosome, nucleus localization, biomolecule release, and system stabilization.

41. The compound of claims 38 or 39 wherein said agent is selected from the group consisting of a cytotoxic agent, a hydrophobic group, a shielding agent, and a targeting ligand.

42. The compound of claim 41 wherein said agent is transferrin.

43. A non-viral delivery system comprising: (a) a biomolecule; (b) a compound coupled to the biomolecule, wherein the compound-biomolecule conjugate comprises the compound of claims 24 or 33.
44. A non-viral delivery system of claim 45 wherein said biomolecule is siRNA
45. A method of treating a mammal, comprising identifying a mammal in need of gene therapy and administering the compound of claim 28 to the mammal, wherein said biomolecule is siRNA that is effective to lower expression of a gene of interest.
46. A method of treating a mammal, comprising identifying a mammal in need of gene therapy and administering the compound of claim 28 to the mammal, wherein said biomolecule is siRNA that is effective to lower expression of a gene of interest.

FIGURE 1



R & R' = H or alkyl

FIGURE 2

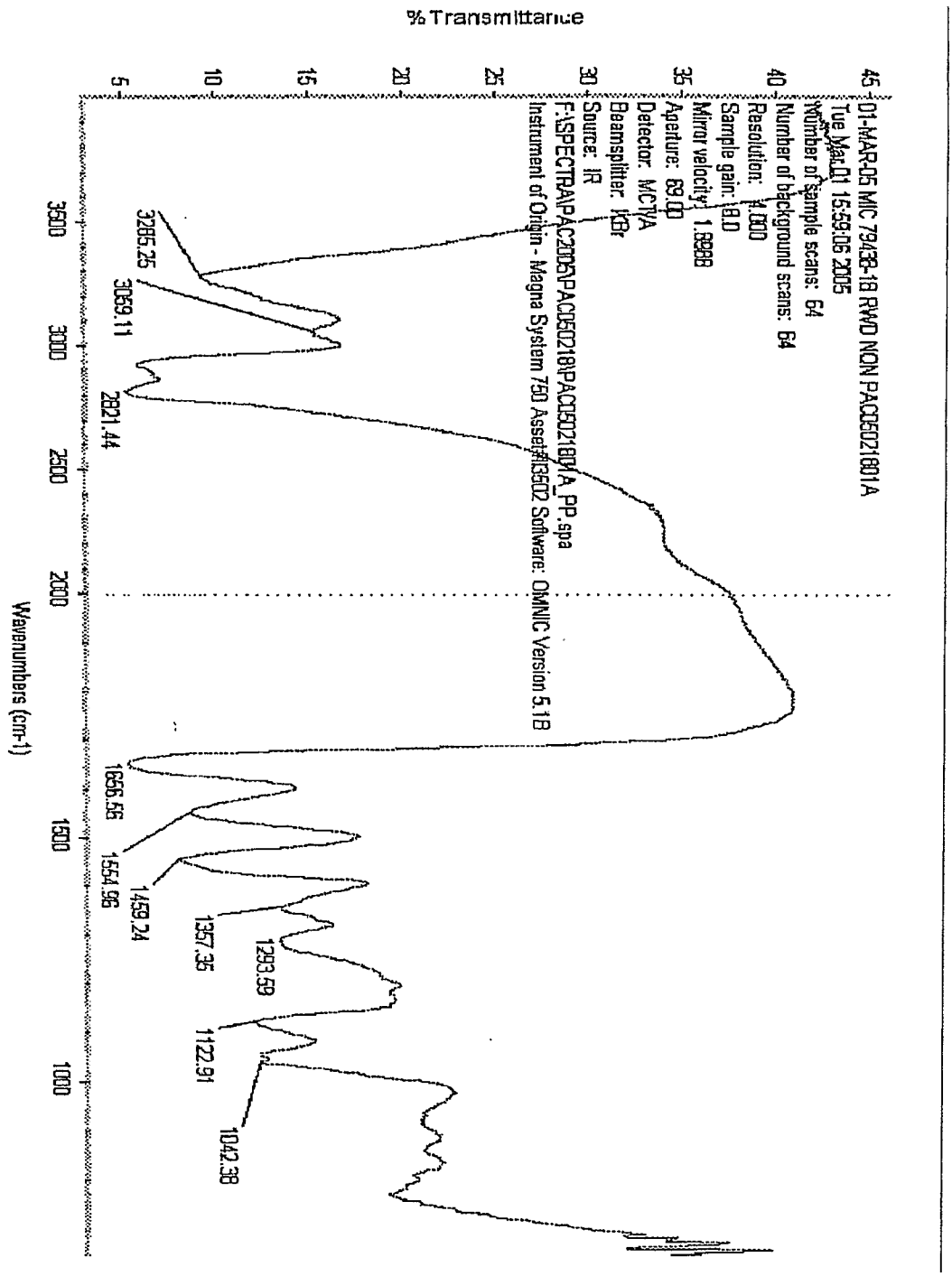

%Transmittance
Wavenumbers (cm-1)
01-MAR-05 MIC 7943B-18 RWD NON PAC050218\01A
Tue Mar 01 15:59:06 2005
Number of sample scans: 64
Number of background scans: 64
Resolution: 4.000
Sample gain: 1.0
Mirror velocity: 1.8988
Aperture: 69.00
Detector: MCT/A
Beamsplitter: KBr
Source: IR
F:\SPECTRA\PAC2005\PAC050218\PAC050218\01A_PP.spa
Instrument of Origin - Magna System 750 Assembly 3502 Software: OMNIC Version 5.1B
3285.25
3069.11
2921.44
1656.56
1554.96
1459.24
1357.35
1293.59
1122.91
1042.38

FIGURE 3a

PEI-800 modified with suberic acid chloride

(possible structure)

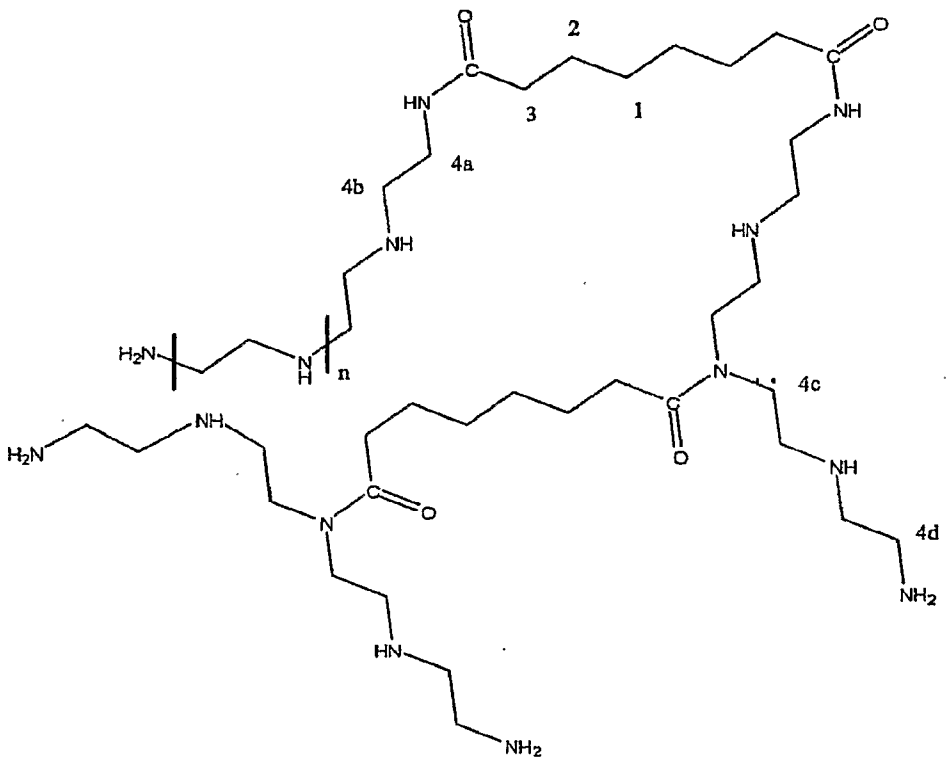


FIGURE 3b

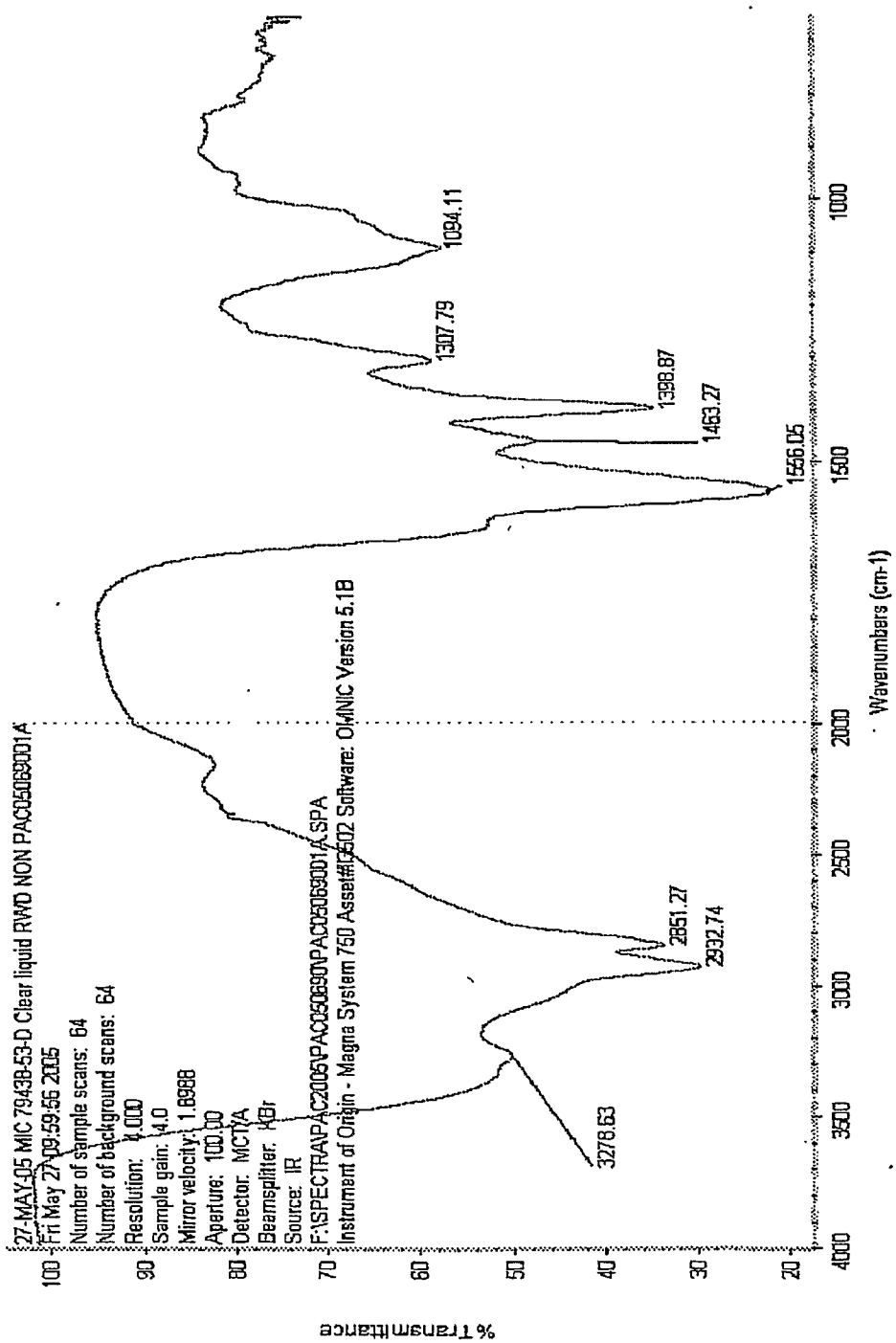

27-MAY-05 MIC 7943B-53-D Clear liquid RWD NON PAC050699001A
Fri May 27 09:59:56 2005
Number of sample scans: 64
Number of background scans: 64
Resolution: 4.000
Sample gain: 4.0
Mirror velocity: 1.8988
Aperture: 100.00
Detector: MCTA
Beamsplitter: KBr
Source: IR
F:\SPECTRA\PAC2005\PAC050699\PAC050699001A.SPA
Instrument of Origin - Magna System 750 Asset#12502 Software: OMNIC Version 5.1B
3278.63
2851.27
2932.74
1556.05
1463.27
1398.87
1307.79
1094.11
% Transmittance
Wavenumbers (cm-1)

FIGURE 4

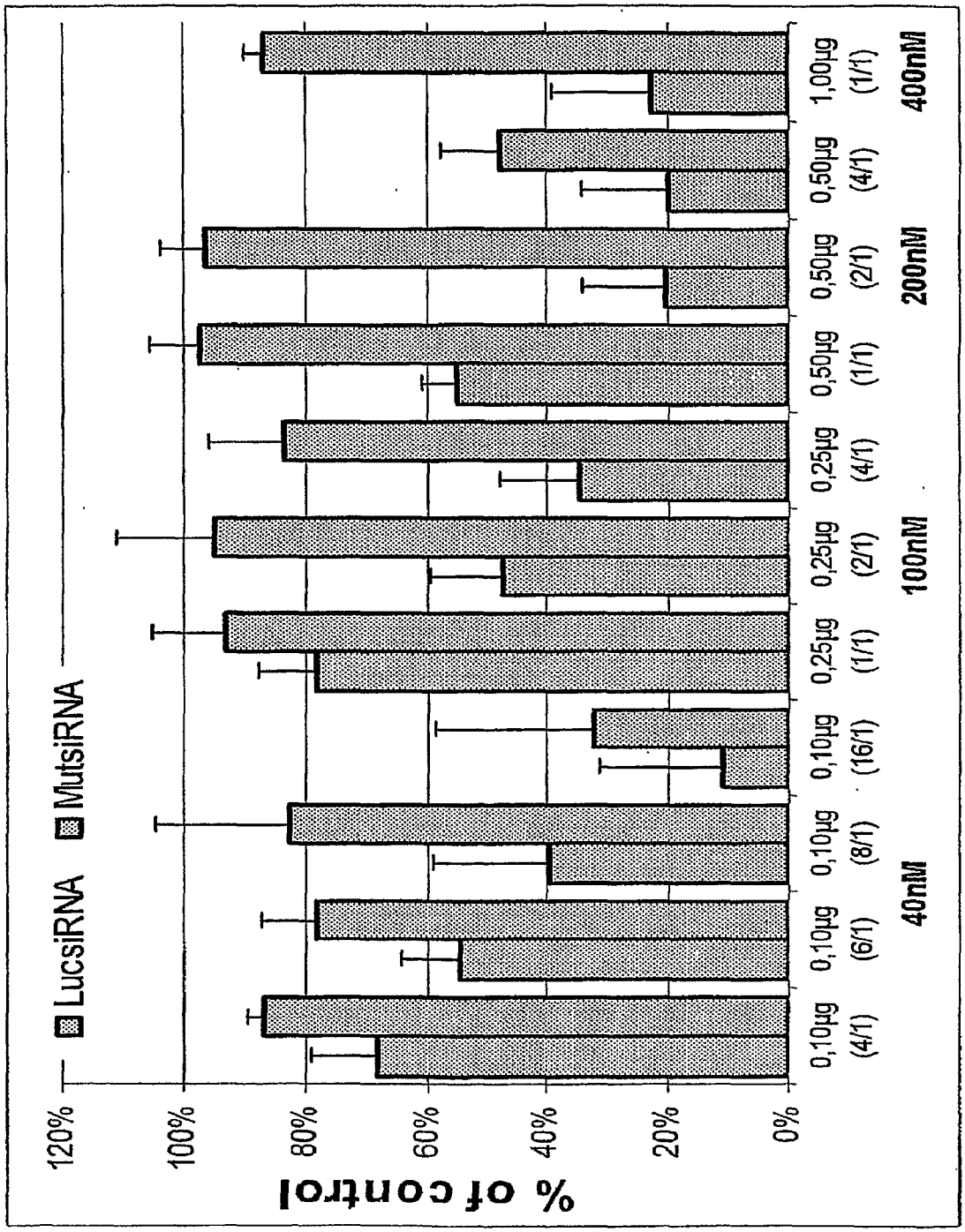

LucsiRNA
MutsiRNA
% of control
120%
100%
80%
60%
40%
20%
0%
0,10µg (4/1)
0,10µg (6/1)
0,10µg (8/1)
0,10µg (16/1)
0,25µg (1/1)
0,25µg (2/1)
0,25µg (4/1)
0,50µg (1/1)
0,50µg (2/1)
0,50µg (4/1)
1,00µg (1/1)
40nM
100nM
200nM
400nM

FIGURE 5

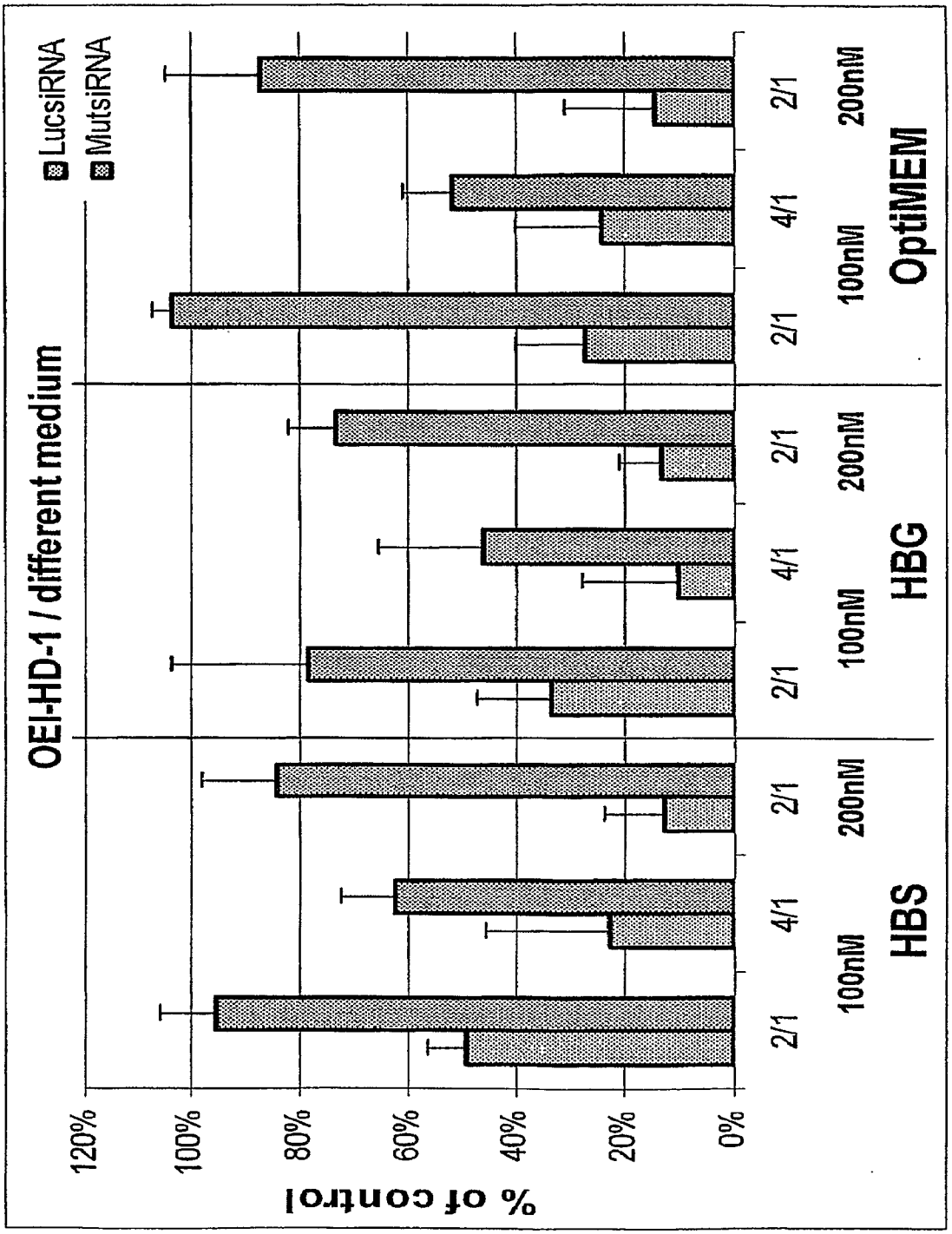

OEI-HD-1 / different medium
LucsiRNA
MutsiRNA
% of control
120%
100%
80%
60%
40%
20%
0%
HBS
HBG
OptiMEM
100nM
200nM
2/1
4/1

FIGURE 6a

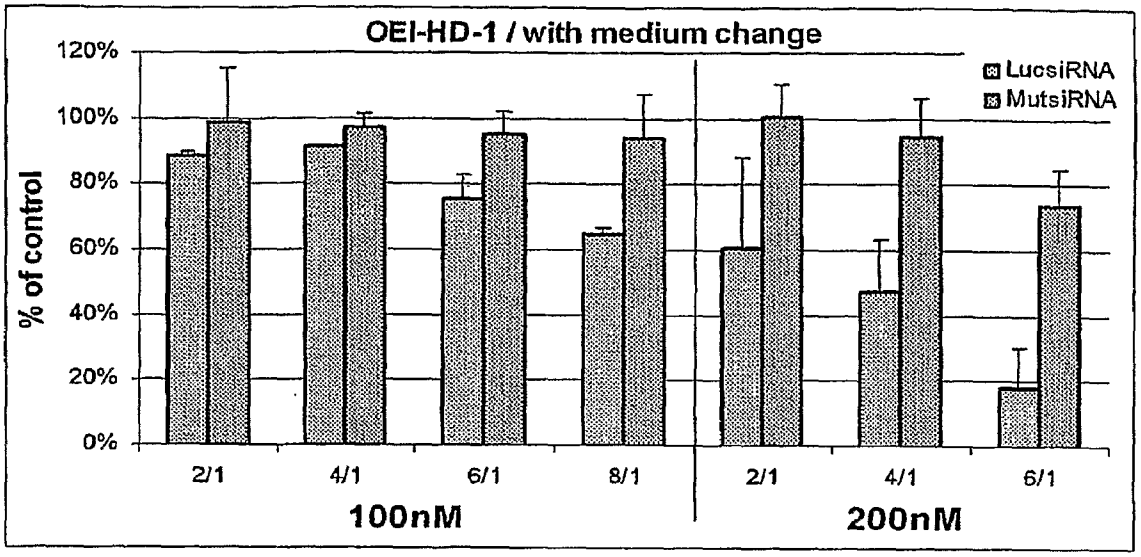


FIGURE 6b

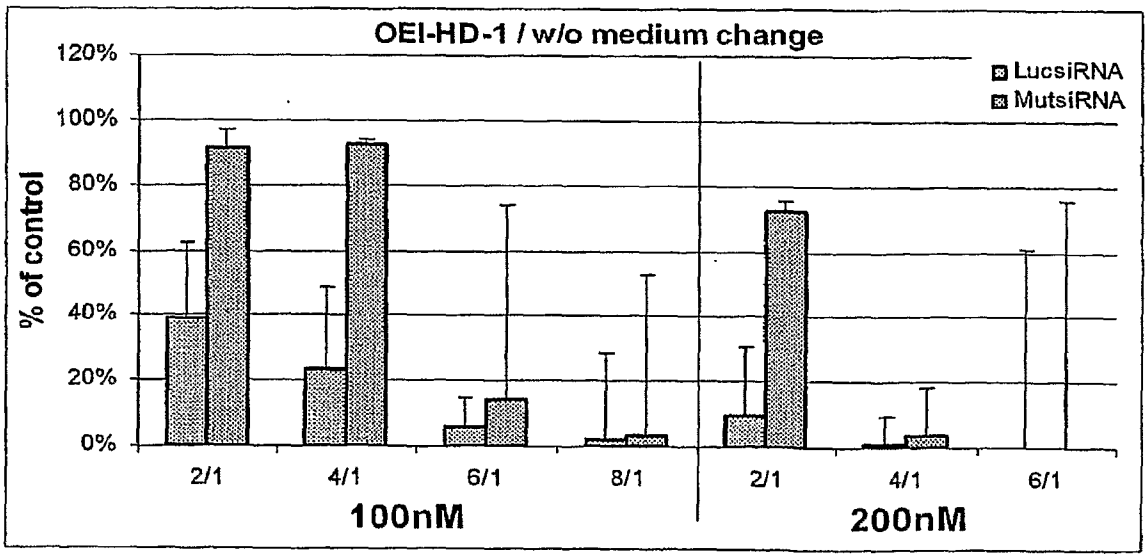


FIGURE 7

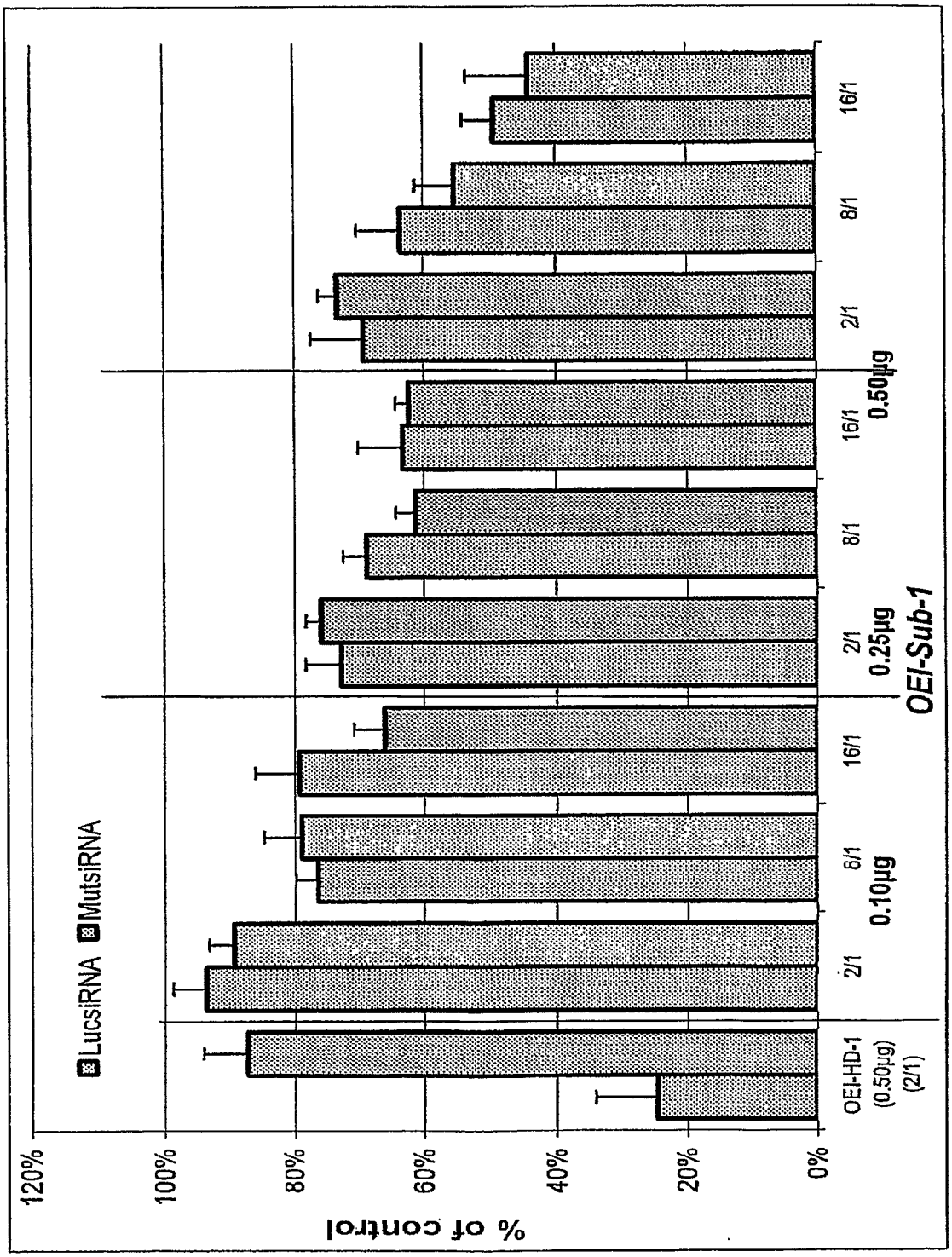

% of control
120%
100%
80%
60%
40%
20%
0%
LucsiRNA
MutsiRNA
OEI-HD-1 (0.50µg) (2/1)
2/1
8/1
16/1
0.10µg
0.25µg
0.50µg
OEI-Sub-1

FIGURE 8

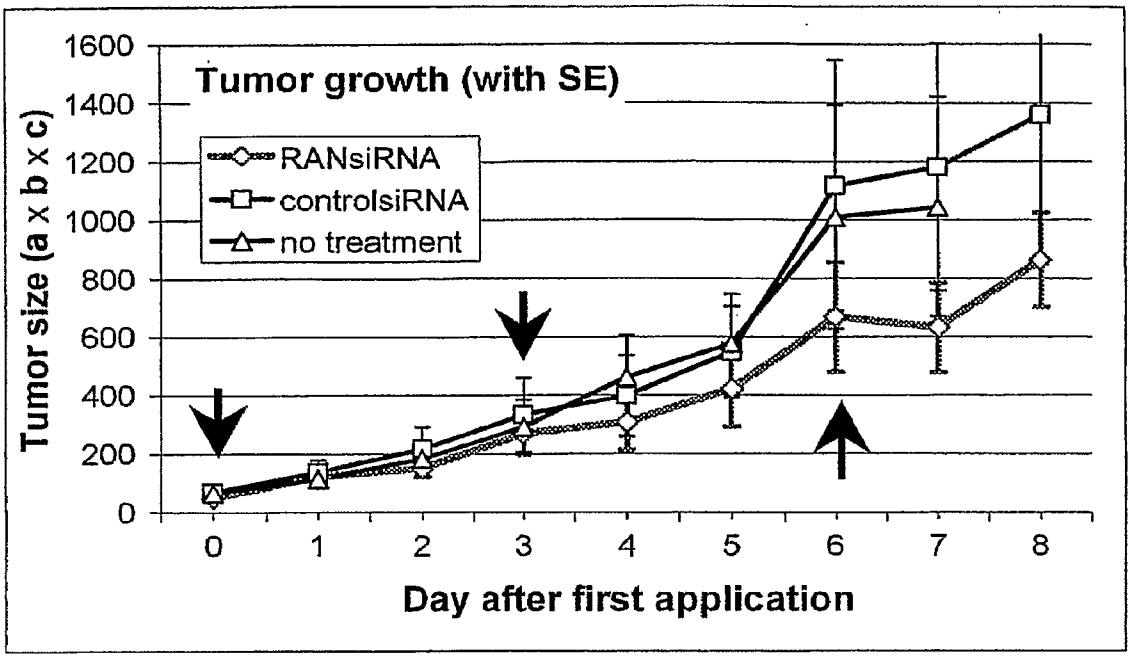
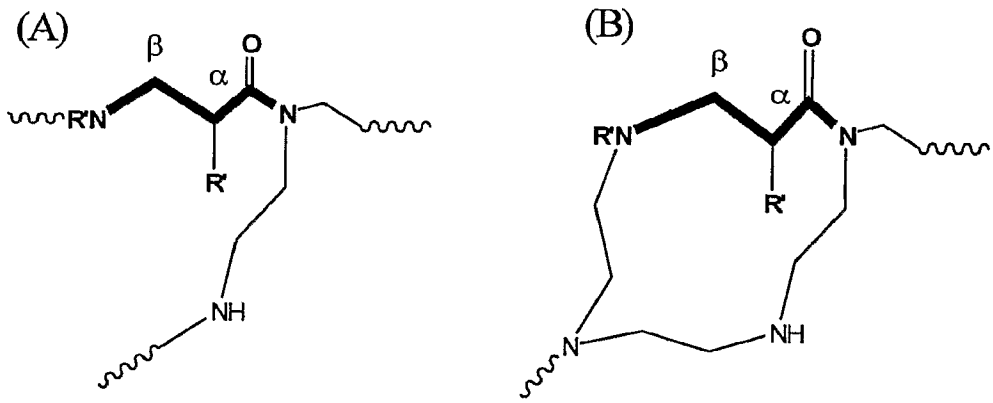

Tumor growth (with SE)
RANsiRNA
controlsiRNA
no treatment
Tumor size (a x b x c)
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
Day after first application

FIGURE 9



The beta-aminopropropionyl amide shown in bold can function as an interchain (A) and/or intrachain (B) linkage (R' = H or alkyl and ∿∿∿ is an extension of a given chain).

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/002024

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/00 A61K47/48 C08G73/00 C08G73/02 C12N15/00
C12N15/11

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)
A61K C08G C12N

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 practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/31025 A2 (MASSACHUSETTS INST TECHNOLOGY [US]) 18 April 2002 (2002-04-18) page 1, line 5 - page 91, line 14; claims 1-101; examples	1-46
A	US 2004/162235 A1 (TRUBETSKOY VLADIMIR S [US] ET AL) 19 August 2004 (2004-08-19) paragraphs [0001] - [0215]; claims 1-19; examples	1-46
A	US 2005/191746 A1 (VAN SANG [US] ET AL) 1 September 2005 (2005-09-01) paragraphs [0001] - [0082]; claims 1-27; examples	1-46

-/--

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

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 April 2007	07/05/2007

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, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kiebooms, Rafaël

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/002024

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/137064 A1 (LEWIS DAVID L [US] ET AL) 15 July 2004 (2004-07-15) paragraphs [0001] - [0209]; claims 1-11; examples	1-46

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2007/002024

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0231025 A2	18-04-2002	CA 2428834 A1	18-04-2002
		EP 1401918 A2	31-03-2004
		JP 2004511596 T	15-04-2004
		JP 2006348310 A	28-12-2006
		US 2005265961 A1	01-12-2005
		US 2004071654 A1	15-04-2004
		US 2002131951 A1	19-09-2002
US 2004162235 A1	19-08-2004	EP 1620560 A1	01-02-2006
		WO 2004076674 A1	10-09-2004
US 2005191746 A1	01-09-2005	EP 1730290 A2	13-12-2006
		WO 2005085458 A2	15-09-2005
US 2004137064 A1	15-07-2004	EP 1597357 A1	23-11-2005
		WO 2004065587 A1	05-08-2004