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(54) Title: OLIGONUCLEOTIDE NON-VIRAL DELIVERY SYSTEMS

(57) Abstract: Low molecular weight low molecular weight chitosan oligomers were able to self- assemble siRNA into nanosized particles, provide protection against enzymatic degradation, and mediate gene silencing that is stable over a long period of time in vitro. The control of structural variables in formulating complexes of siRNA with low molecular weight chitosans provides an efficient alternative delivery system for siRNA *in vitro* and *in vivo*.



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TITLE

OLIGONUCLEOTIDE NON-VIRAL DELIVERY SYSTEMS

FIELD OF THE INVENTION

The present invention relates generally to the fields of nucleic acid delivery and gene
5 expression. In particular, the present invention relates to a new non-viral delivery
system for oligonucleotides, especially small interfering RNA (siRNA).

BACKGROUND OF THE INVENTION

RNA interference (RNAi) is a natural mechanism involving specific down
10 regulation of target gene expression by double-stranded short interfering RNA
(siRNA) [1]. RNAi has increasingly become a well-established tool in functional
genomics and in target screening and validation *in vitro* [2, 3]. More importantly,
the development of siRNA-based drugs holds promise in finding therapies for
complex diseases such as diabetes, cancer, and viral infections [4-7].
15 As for other forms of nucleic acids such as plasmid DNA (pDNA), poor serum
stability, unfavorable pharmacokinetics *in vivo*, and inefficient cellular uptake
remain the main challenges for successful gene silencing applications [8]. One
strategy to partly overcome such problems is the use of chemically modified siRNAs
that exhibit resistance to nuclease degradation as well as improved cellular uptake
20 [9-11]. Another strategy involves the use of polycation-based siRNA formulations.
For instance, cationic lipid-based formulations were shown to be effective for *in*
vitro and *in vivo* delivery of siRNA [12-15]. In contrast, cationic polymers were
initially considered unsuitable for oligonucleotides delivery [16]. However, recent
studies have shown that cationic polymers such as polyethyleneimine (PEI),
25 polyamidoamine (PAMAM) dendrimers and poly-L-lysine (PLL) can be used for

siRNA delivery [17-19]. Contradictory accounts on the efficiency of cationic polymers as delivery systems for oligonucleotides were formulated and delivered under conditions optimized for pDNA. Furthermore, several reports have raised concerns about the *in vivo* toxicity of the above mentioned polycations, which may
5 hamper their future clinical applications [20-22]. Therefore, the search for non-toxic, efficient vectors for siRNA delivery is motivated.

SUMMARY OF THE INVENTION

10 The invention encompasses a composition comprising complexes of: (a) low molecular weight chitosan having a number average Degree of Polymerization (DP_n) in the range between 30 and 300 and where the degree of deacetylation of the low molecular weight chitosan is greater than 90 %; and (b) an oligonucleotide. The composition of claim 1, includes a low molecular weight chitosan obtained from
15 high molecular weight chitosan using chemical or enzymatic methods. The degree of deacetylation of the low molecular weight chitosan is greater than 95 %, and most preferably greater than 99 %. Additionally, the composition essentially has a net positive charge ratio. The low molecular weight chitosan is derivatized with targeting ligands and stabilizing agents. The oligonucleotide comprises a silencing
20 sequence that will express its function when introduced into a host cell. The oligonucleotide is selected from the group consisting of RNA molecules, antisense molecules, Ribozymes, and micro RNAs. The composition of the invention has a pH in the range of 3.5 to 8.0, more preferably in the range of 7.1 to 7.6.
The invention also encompasses method of preparing the inventive composition
25 comprising the steps of:

(a) exposing the low molecular weight chitosan to an aqueous solvent; (b) mixing the aqueous solution of step (a) with an oligonucleotide in an aqueous solvent; and (c) maintaining the pH of the composition in the range of 3.5-8.0, more preferably in the range of 7.1 to 7.6. The invention also contemplates the method of preparing
5 the composition in which after step (b) the volume of the product solution produced in step (b) is reduced to obtain a desired concentration of the composition.

The invention also encompasses a method of administering nucleic acid to a mammal, comprising using the disclosed composition and introducing the composition into the mammal. The method of introducing the composition into the
10 mammal is accomplished by administration to mucosal tissues by pulmonary, nasal, oral, ocular, buccal, sublingual, topical, rectal, or vaginal routes. Alternatively, the composition is introduced into the mammal by administration to submucosal tissues by parenteral routes that are intravenous, intramuscular, intradermal, intracranial, intraspinal, subcutaneous, or intracardiac, or administered to internal organs, blood
15 vessels, or other body surfaces or body cavities exposed during surgery. The inventive method includes administering the disclosed composition to a mammal, whereby the oligonucleotide is capable of expressing its function inside at least one cell of the mammal. The invention also encompasses a method of using the disclosed composition prepared as a medicament for prophylactic or therapeutic
20 treatment of a mammal. These uses include but are not limited to gene therapy, antisense therapy, or genetic vaccination for prophylactic or therapeutic treatment of malignancies, autoimmune diseases, inherited disorders, pathogenic infections and other pathological diseases. Furthermore, the invention encompasses using the disclosed composition as a diagnostic agent for use in *in vivo* or *in vitro* diagnostic
25 methods.

BRIEF DESCRIPTION OF THE DRAWINGS AND THE SEQUENCE LISTING

- Figure 1** shows the physical stability (A) and RNase A protection (B) of siRNA formulations. siRNA complexes formulated with linear DP_n85 chitosans displayed the highest physical stability at both the pH values and at all the charge ratios tested. All the selected polycations were able to protect the siRNA from enzymatic degradation by RNase A. For the agarose gel electrophoresis, 100 ng siRNA was loaded into each well. Complexes were formulated at charge ratios of 30:1 (+/-) and 60:1 (+/-) for chitosan DP_n18 formulations. A charge ratio of 15:1 (+/-) was used for PEI formulations, while complexes of siRNA with lipofectamine 2000 were formulated at a weight ratio of 2:1 (+/-). Representative gels from three independent experiments are shown.
- Figure 2** shows *in vitro* delivery of siRNA in normal HEK 293 cells (A) and in stably luciferase-expressing HEK 293 cells (293-Luc) (B, C). Significant luciferase silencing was achieved when the specific siRNA-Luc was co-transfected with pLuc (A) or pGFP (C) compared with the control untreated cells. Reduced efficiency of luciferase inhibition was obtained when siRNA-Luc was solely delivered to 293-Luc cells (B). Luciferase gene expression was analyzed 48 h after transfection. Chitosans previously optimized for pDNA delivery were used (branched DP_n 34) [27] to form complexes with siRNA formulated at a charge ratio of 10:1. A charge ratio of 5:1 (+/-) was used for PEI complexes, while complexes of siRNA with LF 2000 were formulated at a weight ratio of 2:1 (+/-). The gene expression results are expressed as mean values \pm S.D.; n=4.

Figure 3 shows the influence of the structural variables of linear (A) and branched (B) chitosan complexed with oligonucleotides on the luciferase silencing activity in 293-Luc cells. While the chain length and the charge ratio seemed not to be critical for complexes formed with linear chitosan having chain lengths longer than 34 monomer units (number average degree of polymerization higher than 34 monomer units), efficient luciferase silencing mediated by the branched chitosan complexes required higher charge density in terms of longer chitosan chain lengths and higher charge ratios. A siRNA concentration of 150 nM (100 ng/well) was used. Luciferase gene expression was analyzed 48 h after transfection. Chitosan complexes were formulated at charge ratios of 30:1 (+/-). The gene expression results are expressed as mean values \pm S.D.; n=4.

Figure 4 shows siRNA concentration dependency and relative efficiency of linear low molecular weight chitosans. At lower siRNA concentrations (15-30 ng/well, equivalent to siRNA concentration of 22-44 nM/well), linear DP_n85 low molecular weight chitosans demonstrated the highest potency by knocking down the luciferase expression by 72-95% of the control untreated 293-Luc cells. Luciferase gene expression was analyzed 48 h after transfection. Chitosan complexes were formulated at charge ratios of 30:1 (+/-). The gene expression results are expressed as mean values \pm S.D.; n=4.

Figure 5 illustrates the effect of serum on the luciferase silencing activity. Among the various polycation formulations tested, all linear chitosans and branched DP_n85 retained their luciferase silencing activity in 293-Luc cells in the presence of 10%

serum in the transfection medium. A siRNA concentration of 150 nM was used. Luciferase gene expression was analyzed 48 h after transfection. siRNA complexes were formulated at charge ratios of 30:1 and 15:1 (+/-) for Low molecular weight chitosans and PEI, respectively. Complexes of siRNA with LF2000 were formulated at a weight ratio of 2:1 (+/-). The gene expression results are expressed as mean values \pm S.D.; n=4.

Figure 6 illustrates the cellular toxicity of siRNA formulations. Intracellular dehydrogenase activity (a measure of cell toxicity) was determined by the MTT method directly or 24 h following transfection of 293-Luc cells with various siRNA formulations. In contrast to PEI and LF2000, both the cell morphology and the intracellular dehydrogenase activity were retained following transfection with siRNA complexes formulated with linear DP_n85. Although LF2000 complexes displayed significant toxicity directly following transfection, cell viability was restored after 24 h of transfection. A siRNA concentration of 150 nM was used. siRNA complexes were formulated at charge ratios of 30:1 and 15:1 (+/-) for low molecular weight chitosans and PEI, respectively. Complexes of siRNA with LF2000 were formulated at a weight ratio of 2:1 (+/-). The gene expression results are expressed as mean values \pm S.D.; n=4-5.

20

Figure 7 shows the time course of the luciferase silencing in 293-Luc cells (A, B, C) and in stably luciferase-expressing SKOV-3 cells (D) *in vitro*. In both 293-Luc and SKOV-3-Luc cells, linear chitosan with DP_n85 displayed the best luciferase silencing kinetics in terms of earlier onset and sustained luciferase silencing that lasted for 5 days, suggesting a stable release of intact siRNA intracellularly. A

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siRNA concentration of 44 nM (30 ng/well) was used. siRNA complexes were formulated at charge ratios of 30:1 and 15:1 (+/-) for low molecular weight chitosans and PEI, respectively. Complexes of siRNA with LF2000 were formulated at a weight ratio of 2:1 (+/-). The gene expression results are expressed as mean values
 5 \pm S.D.; n=4.

BRIEF DESCRIPTION OF THE SEQUENCE LISTING

SEQ ID NO. 1: siGL3 (sense, 5'-CUUACGCUGAGUACUUCGAdTdT-3';

10 SEQ ID NO. 2: antisense, 5'-UCGAAGUACUCAGCGUAAGdTdT-3') is an unmodified siRNA duplex that targets the luciferase gene (siRNA-Luc), ordered from MedProbe (Lund, Sweden) [28].

SEQ ID NO.3: A mismatching siRNA; siCONTROL non-targeting siRNA #1 (siCON1; sense, 5'-UAGCGACUAAACACAUCAAUU-3';

15 SEQ ID NO. 4: antisense, 5'-UUGAUGUGUUUAGUCGCUAUU-3'), was ordered from Dharmacon Research, Inc. (Lafayette, CO).

DETAILED DESCRIPTION OF THE INVENTION

Chitosans, a family of linear binary polysaccharides comprised of (1-4) linked 2-amino-2-deoxy- β -D-glucose (GlcN) and the *N*-acetylated analogue 2-acetamido-2-deoxy- β -D-glucose (GlcNAc) are biocompatible cationic polymers, and have been
 20 shown to be suitable for plasmid pDNA gene delivery [23-27]. Quite effective gene delivery *in vitro* and *in vivo* has been obtained with linear and branched chitosan oligomers of well-defined molecular weight distributions [25, 27]. The chitosan-oligomer-based complexes had improved physical properties including reduced
 25 viscosity and were less prone to aggregation. These complexes also possessed

improved efficiency including improved cellular uptake, early onset, and high levels of *in vivo* gene expression.

- In the instant invention, the potential low molecular weight (7-17 kDa), essentially fully deacetylated (greater than 99% deacetylated) chitosans in novel siRNA delivery systems is realized. Using lipofectamine 2000 (LF2000) and PEI (linear and branched) as controls, the physical stability and the resistance against RNase degradation of siRNA complexes formulated with low molecular weight chitosans was investigated. In most reports on siRNA delivery, a co-transfection method is used to incorporate a non-physiologically relevant target or an irrelevant pDNA in the siRNA formulations. Therefore, we first examined the effect of such incorporation on the efficiency of siRNA delivery by various polycations in stably luciferase-expressing cell lines *in vitro*. We then investigated formulation and structure in complexes of siRNA formulation and low molecular weight chitosans. More specifically, the role of the structural variables of low molecular weight chitosans (chain length and branching), the formulation parameters (charge ratios, siRNA concentrations), and the effect of serum were studied and correlated to the efficiency of the gene silencing activity *in vitro*. The cellular toxicity of various siRNA formulations was compared as was the *in vitro* kinetics of luciferase gene silencing.
- 20 This work demonstrates the potential of low molecular weight chitosan as novel delivery systems for small interfering RNA (siRNA). Using polyethyleneimine (PEI) and lipofectamine 2000 (LF2000) as controls, chitosan of various chain lengths were complexed with siRNA and their physical stability and protection against enzymatic degradation were examined. The cellular toxicity and luciferase gene silencing activity of the siRNA complexes were investigated in stably

luciferase-expressing 293 cells (293-Luc) *in vitro*. The effects of chitosan structural variables as well as the formulation parameters on the luciferase silencing activity of siRNA complexes were also studied. Low molecular weight chitosans were able to complex siRNA into physically stable, nanoparticles (34-86 nm) that provided
5 protection against RNase degradation. The importance of a higher number of positive charges provided by longer chitosan chain and/or higher charge ratios between of low molecular weight chitosans and siRNA was shown for mediating the highest luciferase silencing activity *in vitro*. Unlike PEI and LF2000, siRNA complexes formulated with low molecular weight chitosans retained their luciferase
10 silencing activity transfection medium contained 10 % serum. Low molecular weight chitosans also displayed minimal cellular toxicity compared to PEI and LF2000. Low molecular weight chitosans having a number-average degree of polymerization (DP_n) of 85 monomer units ($DP_n 85$) required a siRNA concentration as low as 44 nM to obtain 95% silencing of the luciferase gene expression that was
15 sustained for 5 days in 293-Luc cells. Taken together, our findings demonstrate low molecular weight chitosans as an efficient alternative delivery system for siRNA. We previously reported that essentially fully deacetylated low molecular weight chitosans of quite short chain lengths (18-34 monomer units) were optimal for the delivery of pDNA *in vitro* and *in vivo* [25, 27]. In the instant work, we report on the
20 structure-property relationships of essentially fully deacetylated chitosan oligomers as siRNA delivery systems *in vitro*. For this purpose, linear and trisaccharide-substituted (branched) chitosan oligomers of various chain lengths were selected. We found that, in contrast to pDNA delivery, linear chitosan oligonucleotides longer than 34 monomer units formed physically stable complexes with siRNA and
25 mediated the highest luciferase silencing activity in luciferase-expressing HEK 293

(293-Luc) cells *in vitro*. Apparently, the structure-property relationships are quite different between siRNA and pDNA. This difference may be explained in that siRNA molecules are shorter, less flexible, and have a lower negative charge density compared to pDNA. Therefore, the complexation of siRNA into physically stable and efficient nanoparticles requires stronger ionic interaction with polycations [18, 33].

The particle size of the siRNA complexes formulated with essentially fully deacetylated low molecular weight chitosans in our work (less than 100 nm) was unexpectedly much smaller than those previously reported for lipids, PLL, and high molecular weight chitosans (85 % deacetylation) [33, 34]. This can be attributed to the higher charge density on the essentially fully deacetylated chitosan backbone. In addition, the enhanced solubility and reduced viscosity of the low molecular weight chitosan may contribute to the small particle sizes of the siRNA complexes [35]. In agreement with previous findings, the co-transfection of the siRNA-Luc together with an irrelevant pDNA (pGFP) as a single package resulted in a significant luciferase silencing activity in 293-Luc cells *in vitro* [17]. However, we showed that the gene silencing activity was significantly compromised when the pDNA was excluded from the siRNA formulations. We conclude, therefore, that the presence of pDNA, a macromolecule with a high negative charge density significantly contributed to the efficiency of siRNA complexation with various polycations via improved cooperative interactions.

We also showed that a high charge density in terms of longer chitosan chains and/or higher charge ratios will not only yield physically stable complexes but also obtain the most efficient gene silencing *in vitro*. Our findings are in good agreement with previous results reported for PAMAM dendrimers, where higher generation

numbers, higher charge ratios, and higher siRNA concentrations (100 nM) were required for better complexation and gene silencing activity obtained by siRNA/dendrimers complexes [18]. A similar high concentration of siRNA was also recommended for LF2000-based formulations [12]. In this instant invention, the

5 most efficient low molecular weight chitosans (linear DP_n85) required a siRNA concentration as low as 44 nM to obtain more than 95% silencing of the luciferase gene expression in 293-Luc cells.

Moreover, under the experimental conditions in this work, siRNA complexes formulated with long, linear low molecular weight chitosans retained their luciferase

10 silencing activity in the presence of 10% serum in the transfection medium. The most likely reason for the retained silencing activity may be that these complexes, in contrast to PEI and LF2000, can resist aggregation in such relatively high serum concentration, which may reflect an enhanced colloidal stability. The finding that shorter and branched chitosan oligomers demonstrated reduced gene silencing

15 activity could be explained by the impaired siRNA complexation as a result of the reduced charge density and the steric hindrance of the charge interaction between the chitosan backbone and siRNA.

In agreement with the minimal cellular toxicity reported of siRNA complexes

20 formulated with high molecular weight chitosans, we showed in this work that 293-Luc cells retained their intracellular dehydrogenase activity following transfection with DP_n85 low molecular weight chitosans even when a high siRNA concentration (150 nM) was used in the formulation [33]. Low molecular weight chitosans displayed a much lower cellular toxicity compared to previously published results on

PAMAM dendrimers where the use of siRNA concentrations of 50-100 nM has lead to a significant reduction in cell viability (60-50%).

Finally, siRNA complexes formulated with linear low molecular weight chitosans
5 displayed earlier onset and sustained luciferase silencing activity compared to those previously reported for PEI [17]. The improved kinetics of DP „85 chitosan oligonucleotides is assumed to be a result of the improved cellular uptake of the small, nano-sized siRNA complexes and the sustained release of intact siRNA intracellularly.

10

2. Materials and methods

2.1. Materials

15 A GMP-grade plasmid (gWiz™) containing a cytomegalovirus promoter and a firefly luciferase (pLuc) or green fluorescence protein (pGFP) was purchased from Aldevron, Fargo, ND, USA. Lipofectamine 2000 (LF2000) was purchased from Invitrogen. Linear PEI; ExGen 500 (molecular weight of 22 kDa) was purchased from Ferementas, Germany. Branched PEI (molecular weight of 25 kDa) was
20 purchased from Aldrich Sweden, Stockholm, Sweden.

2.2. siRNA duplexes

SEQ ID NO. 1: siGL3 (sense, 5'-CUUACGCUGAGUACUUCGAdTdT-3';

SEQ ID NO. 2: antisense, 5'-UCGAAGUACUCAGCGUAAGdTdT-3') is an unmodified siRNA duplex that targets the luciferase gene (siRNA-Luc), ordered from MedProbe (Lund, Sweden) [28].

SEQ ID NO.3: A mismatching siRNA; siCONTROL non-targeting siRNA #1

5 (siCON1; sense, 5'-UAGCGACUAAACACAUCAAUU-3';

SEQ ID NO. 4: antisense, 5'-UUGAUGUGUUUAGUCGCUAUU-3'), was ordered from Dharmacon Research, Inc. (Lafayette, CO).

2.3. Low molecular weight chitosans

10 Fully de-*N*-acetylated (degree of deacetylation > 99.8 %; $F_A < 0.001$) linear and 7% trisaccharide-substituted low molecular weight chitosans (branched chitosans) of various chain lengths were prepared and characterized as described [25,29]. Low molecular weight chitosans having number-average degrees of polymerization (DP_n) of 34, 50 and 85 monomer units were used throughout. The chain length
15 distributions were analysed by size exclusion chromatography with a multi-angle laser light scattering (SEC-MALLS).

2.4. Cells

The human embryonic kidney cell line HEK 293 (293 cells) was obtained from
20 ATCC, Rockville, MD, USA. Stably luciferase-expressing HEK 293 (293-Luc cells) that express firefly luciferase was a gift from Dr. Paavo Honkakoski, Department of Pharmaceutics, University of Kuopio, Finland [30]. Stably luciferase-expressing ovarian carcinoma cell line (SKOV-3-Luc) was also a gift from Dr. Achim Aigner, Department of Pharmacology and Toxicology, Philipps-

University Marburg, Germany [17]. All cells were maintained according to the suppliers' recommendations.

2.5. Formulation of siRNA complexes

5 Chitosan stock solutions (0.2 mg/ml) were prepared by dissolving chitosan in sterile MilliQ water at pH 6.2 followed by sterile filtration. Chitosan complexes were formulated by adding chitosan and then siRNA stock solutions or siRNA/pDNA (in case of co-transfection) to sterile MilliQ water during intense stirring on a vortex mixer (Heidolph REAX 2000, level 4, Kebo Lab, Spånga, Sweden) as described

10 [25]. The following amounts of the different chitosans were used per µg pDNA or siRNA to prepare chitosan complexes at a charge ratio of 1:1 (+/-): 0.58 µg of linear chitosans, 0.69 µg of chitosans substituted with 7% A-A-M (branched chitosans) [25, 27]. siRNA complexes of PEI were prepared by adding PEI solutions to siRNA stock solutions or to siRNA/pDNA (in the case of transfection) during intense

15 stirring on a vortex mixer as described [31]. The formulations were left for approximately 10 min. at room temperature before transfection. To form LF2000 complexes, siRNA stock solutions or siRNA/pDNA (in case of co-transfection) were added to chitosan solutions during intense stirring on a vortex mixer. Both siRNA and LF2000 solutions were diluted with the transfection medium OptiMEM I.

20 LF2000 complexes were left for approximately 30 min. at room temperature before transfection. Based on preliminary experiments, a charge ratio of 15:1 (+/-) for PEI complexes and a weight ratio of 2:1 (+/-) for LF2000 complexes were selected and used throughout (data not shown).

25 2.6. Gel retardation assay

The physical stability of the siRNA complexes was studied using the agarose gel retardation assay. 4 % agarose (MetaPhor® Agarose, Cambrex Bio Science Rockland, Inc., Rockland, ME, USA) in 40 mM TAE buffer was used as described [25]. Protection of complexed siRNA against enzymatic degradation was studied after incubating the complexes with 1.5 U RNase A (Ambion, UK) for 30-90 minutes as described [18]. After the incubation, the complexes were dissociated with heparin (5 mg/ml) and the integrity of siRNA was examined using the agarose retardation assay. siRNA obtained from the stock solution was used as control.

10 2.7. Size measurements of chitosan polyplexes

The size of the complexes was determined by photon correlation spectroscopy using Nanosizer ZS (Malvern Instruments, Malvern, UK) as described [25]. The complexes were prepared at a siRNA concentration of 5 µg/ml in MilliQ water. All measurements were performed at 25 °C.

15

2.8. In vitro transfection experiments

Twenty-four hours before the transfection experiments, HEK 293 (293 , 293-Luc) cells and SKOV-3-Luc cells were seeded in 96-well tissue culture plates (Costar, Cambridge, UK) to get cell confluency of 80-90% on the day of transfection.

20 Transfections were carried out at pH 7.4 in serum-free medium (OptiMEM I Reduced Serum Media, Gibco/BRL Life Technologies AB, Täby, Sweden) or in the presence of 10% serum (FBS). Isotonicity (300 mOsm/kg) was obtained by the addition of mannitol. The cells were washed with pre-heated OptiMEM and 50 µl of the siRNA complex formulations was added to each well. In co-transfection experiments, 0.33 µg pDNA (pLuc or pGFP) per well was used. A mismatch

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(control) siRNA was included in all *in vitro* experiments. After 5 h incubation, the formulations were removed and 0.2 ml of fresh culture medium was added. The medium was changed every second day for experiments that exceeded two days. At pre-specified time points, ranging from 24 to 120 hours after transfection, the cells were washed with pre-heated PBS (pH 7.4), and lysed with luciferase lysis buffer (Promega, Madison, WI). The luciferase gene expression was then measured with a luminometer (Mediators PhL, Vienna, Austria). The amount of luciferase expressed was determined from a standard curve prepared with firefly luciferase (Sigma, St Louis, MO).

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2.9. Intracellular dehydrogenase activity (MTT method)

The effect of various siRNA formulations on the intracellular dehydrogenase activity (a measure of cellular toxicity) in 293-Luc cells was evaluated by the MTT method as described [32]. Briefly, 293-Luc cells were transfected as described above. After 5 h of transfection, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, Deisenhofen, Germany) solution in phosphate buffered saline (PBS) was added. After 4 h, the formazan crystals were dissolved by adding 100 µl of acid-isopropanol (0.04 M HCL in isopropanol). Absorption was measured at 570 nm with a background correction at 690 using a plate reader (TECAN Safire², Tecan Austria GmbH, Grödig, Austria). The instrument was set to 0 absorbance with culture medium treated in the same way. Intracellular dehydrogenase activity of the treated cells was related to that of the control (untreated) cells, and calculated from the following equation:

15
20

$$\% \text{ Relative Intracellular Dehydrogenase Activity} = [A (\text{test}) * 100 / A (\text{control})],$$

where A (test) and A (control) are the absorbance values of the treated and control cells, respectively. In another setup, the cells were allowed to grow in culture medium for 24 h after transfection. Then, they were treated with the MTT reagent to test delayed toxicity of various formulations.

5

2.10. Data Analysis

The experiments were performed on a minimum of two occasions using quadruplicate samples. All data are expressed as mean values \pm standard deviation. Statistical differences between mean values were investigated using ANOVA.

10 Differences between group means were considered significant at $p < 0.05$.

Physical Stability and Enzymatic Protection

We first investigated the ability of linear and branched chitosan oligomers of various chain lengths to form stable complexes with siRNA in the gel retardation assay. Only complexes formulated with the longer, linear oligonucleotides with higher charge ratios retained the siRNA at pH 8.0, which is a commonly used pH for electrophoresis buffers (Fig 1A). The linear low molecular weight chitosans having a number-average degree of polymerization (DP_n) of 85 monomer units (DP_n85) provided the highest physical stability at all the charge ratios tested. In contrast, when the pH of the gel buffer was lowered to 7.4, all the low molecular weight chitosans tested were able to form stable complexes with the siRNA. The reduced physical stability of siRNA formulations of branched and shorter chitosan oligomers at elevated pH values suggests that a higher charge density of the polycation and a stronger interaction with the siRNA are required to form physically stable

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complexes compared to previously optimized pDNA complexes formulated with low molecular weight chitosans [25, 27].

Since enzymatic degradation can be a limiting factor for gene silencing activity, we also investigated the ability of the selected low molecular weight chitosans to protect
5 siRNA from enzymatic degradation by RNase A (incubation periods of 30-90 minutes were used).

In accordance with gene delivery requirements, all low molecular weight chitosans tested as well as the positive controls (PEI and LF2000) provided protection against enzymatic degradation compared to naked siRNA, which was completely degraded
10 by RNase A (Fig 1B). A longer incubation time with heparin (2 h) was needed to disrupt the complexes formulated with linear DP_n85 and LF2000 reflecting enhanced physical stability compared to the other polycations tested (data not shown).

Particle Size

15 Since the particle size of siRNA formulations can greatly affect their tissue distribution and cellular uptake, we therefore investigated the particle size of siRNA complexes formulated with low molecular weight chitosans. Table 1 shows that low molecular weight chitosans self-assembled with siRNA into nanosized particles (34-86 nm). The size of the resulting particles was dependent on the +/- charge ratios of
20 the components. While small particle sizes (34- 46 nm) were obtained at the lowest charge ratio 10:1 (+/-), the use of higher charge ratios resulted in relatively larger particles sizes (61-86 nm). Particle size was determined by photon correlation spectroscopy.

25

TABLE 1

	A/P 10	A/P 30	A/P 60
DPn 18	38.1 ± 1.2	54.5 ± 0.1	62.5 ± 3.7
DPn 34	37.7 ± 0.5	53.1 ± 0.9	68.2 ± 3.0
DPn50	34.0 ± 0.5	51.7 ± 1.2	63.6 ± 1.1
DPn85	37.4 ± 1.1	51.6 ± 3.3	61.0 ± 2.5
DPn 34-AAM-7%	38.5 ± 0.8	50.8 ± 0.4	68.9 ± 2.7
DPn85-AAM-7%	46.0 ± 2.5	69.9 ± 1.7	86.1 ± 2.9

Comparison of the silencing of co-transfected and stably-expressed target

- 5 Since in most *in vitro* experiments siRNA molecules are delivered simultaneously with their pDNA targets (co-transfection), we first tested the efficiency of low molecular weight chitosans to deliver a package of pDNA coding for firefly luciferase reporter (pLuc) together with a siRNA that targets the same reporter sequence (siRNA-Luc) using a mismatch (non-silencing) siRNA as a control. The
- 10 transfection was carried out in 293 cells (not expressing luciferase reporter) under optimized transfection conditions of pDNA dose, most efficient low molecular weight chitosans and charge ratios, which were optimized for pDNA delivery in our lab [27]. siRNA-Luc delivered by branched DP_n34 chitosan oligomers or LF2000 lead to a significant knockdown in luciferase expression (85-90%) compared to the
- 15 control formulations (only pLuc) (Fig 2A). A non-significant inhibition in luciferase expression was observed with the mismatch siRNA for both delivery systems.

However, when the siRNA-Luc was instead exclusively delivered under the same conditions by the selected polycations to 293-Luc cells that stably express luciferase (a case which is more relevant to gene silencing applications), a very low luciferase silencing activity was obtained, and higher siRNA concentrations were required to obtain a significant silencing in luciferase expression (Fig 2B).

To investigate if the co-transfection technique could have an effect on the gene silencing activity in the 293-Luc cells, an irrelevant plasmid (pGFP) was incorporated in the same siRNA-Luc formulations tested above. Similarly to the pattern obtained in 293 cells, branched chitosan oligomers, PEI and LF2000 mediated a significant knockdown (40-85%) in luciferase expression with the lowest siRNA concentrations (1-30 ng/well, equivalent to 1.5-40 nM/well) (Fig 2C). The incorporation of pDNA in the siRNA formulations (co-transfection) resulted in a positive effect on the gene silencing activity. These results suggest that the formulation requirements for siRNA delivery differ from those of pDNA, and there are important parameters to be characterized for successful siRNA delivery by polycations *in vitro*.

Influence of the structural variables of low molecular weight chitosans and formulation parameters on the gene silencing activity

a) Chain length, backbone branching and charge ratios

In order to examine the effect of chitosan structure and the formulation parameters on siRNA delivery by low molecular weight chitosans, we first tested the influence of chain length, branching and charge ratio on the *in vitro* silencing of luciferase expression mediated by siRNA complexes in 293-Luc cells. For linear chitosans, chitosans with chain lengths longer than 34 monomer units mediated significant

luciferase silencing independently on the charge ratio (Fig 3A). The luciferase silencing mediated by complexes formulated with the branched chitosan oligomers was dependent on both the charge ratio and the chain length (Fig 3B). For the longer branched chitosan oligomers, the higher charge ratios can compensate for the negative effect of the branching (substitution) of the chitosan backbone. These results emphasize that a high charge density of low molecular weight chitosans will not only yield physically stable complexes but also efficient siRNA formulations *in vitro*.

10 ***b) siRNA concentration and the relative efficiency of linear low molecular weight chitosans***

In the next step, the effect of the siRNA concentration on the luciferase silencing activity of complexes formulated with various linear low molecular weight chitosans (at constant charge ratios) was investigated in 293-Luc cells. With the lowest siRNA concentrations (15-50 ng/well, equivalent to 22-73 nM/well), linear DP_n85 complexes demonstrated the highest potency by knocking down the luciferase expression by 72-95% of the control untreated cells (Fig 4). With the exception of DP_n18, complexes of the tested linear chitosans showed comparable luciferase silencing profiles when the siRNA concentration was increased from 70 to 300 ng/well (103-440 nM/well). The higher potency of the complexes formulated with linear, longer chain low molecular weight chitosans supports the role of the physical stability of siRNA formulations in achieving the highest gene silencing activity.

***In vitro* transfection in the presence of serum**

We also investigated the effect of serum in the transfection medium on the efficiency of various siRNA formulations. siRNA complexes formulated with linear chitosans having a range of DP values of 34-85 monomer units and branched DP_n85 chitosan oligomers retained their gene silencing activity in 293-Luc cells in the presence of 10% serum (Fig 5). In contrast, the gene silencing activity of siRNA complexes formulated with PEI and LF2000 was compromised. One possible reason for the compromised efficiency is particle aggregation during transfection.

Cellular toxicity *in vitro*

To ensure that the higher luciferase silencing efficiency was not a result of increased cellular toxicity, we investigated the effect of various polycation complexes formulated with a relatively high concentration of siRNA (150 nM) on the cellular morphology and the intracellular dehydrogenase activity using the MTT method. After 5 h of transfection, siRNA complexes formulated with linear DP_n85, no effect on cell morphology and intracellular dehydrogenase activity (a measure of cellular toxicity) was observed (Fig 6). In contrast, a significant toxic effect was observed with PEI and LF2000, with the highest toxicity for linear PEI. While cells treated with LF2000 complexes restored their intracellular dehydrogenase activity after 24 h, cells treated with PEI displayed a further reduction in dehydrogenase activity after 24 h. These results demonstrate that the acute cellular toxicity of linear DP_n85 low molecular weight chitosans was lower than that of PEI and LF2000 even when relatively high concentrations of siRNA were used.

Kinetics of RNAi *in vitro*

Finally, the time course of luciferase silencing following the delivery of siRNA-Luc to 293-Luc cells was investigated. Low molecular weight chitosans having a range

of DP values of 34-85 monomer units, PEI and LF2000 were tested. DP_n 18 chitosans were not included because they were less efficient than the longer chitosans (Fig 4). siRNA complexes formulated with linear DP_n85 mediated an early onset of luciferase silencing where a significant effect was detected after 1 day (70%), reaching a maximum (92%) after 2 days (Fig. 7A). The gene silencing activity was sustained for 5 days suggesting stable release of intact siRNA intracellularly. Branched chitosan oligomers demonstrated less efficient luciferase silencing kinetics compared to their linear counterparts (Fig 7B). LF2000 and PEI showed a lower gene silencing effect than that mediated by low molecular weight chitosans (Fig 7C). The luciferase silencing kinetics in another cell line, SKOV-3-Luc, gave results similar to those in 293-Luc cells (Fig 7D).

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CLAIMS

What is claimed is:

1. A composition comprising complexes of:

- (a) low molecular weight chitosan having a Degree of Polymerization (DP_n) in the range between 30 and 300 and where the degree of deacetylation of the low molecular weight chitosan is greater than 90 %; and
- (b) a oligonucleotide.

2. The composition of claim 1, wherein the low molecular weight chitosan is obtained from high molecular weight chitosan using chemical or enzymatic methods.

3. The composition of claim 1, where the degree of deacetylation of the low molecular weight chitosan is greater than 95 %.

4. The composition of claim 3, wherein the degree of deacetylation of the low molecular weight chitosan is greater than 99%.

5. The composition of claim 1, wherein the composition essentially has a net positive charge ratio.

6. The composition of claim 1 wherein the low molecular weight chitosan is derivatized with targeting ligands and stabilizing agents.

7. The composition of claim 1, wherein the oligonucleotide comprises a silencing sequence that will express its function when introduced into a host cell.

8. The composition of claim 7, wherein the oligonucleotide is selected from the group consisting of RNA molecules, antisense molecules, Ribozymes, and micro RNAs.

9. The composition of claim 8, wherein the composition has a pH in the range of 3.5 to 8.0.
10. The composition of claim 9, wherein the composition has a pH in the range of 7.1 to 7.6.
11. A method of preparing the composition of claim 1, comprising the steps of:
- (a) exposing the low molecular weight chitosan to an aqueous solvent;
 - (b) mixing the aqueous solution of step (a) with an oligonucleotide in an aqueous solvent; and
 - (c) maintaining the pH of the composition in the range of 3.5-8.0.
12. The method of claim 11 further comprising reducing the volume of the product solution produced in step (b) to obtain a desired concentration of the composition.
13. A method of administering nucleic acid to a mammal, comprising introducing the composition of claim 1 into the mammal.
14. The method of claim 13, wherein the composition is introduced into the mammal by administration to mucosal tissues by pulmonary, nasal, oral, ocular, buccal, sublingual, topical, rectal, or vaginal routes.
15. The method of claim 13, wherein the composition is introduced into the mammal by administration to submucosal tissues by parenteral routes that is intravenous, intramuscular, intradermal, intracranial, intraspinal, subcutaneous, or intracardiac administration, or to internal organs, blood vessels, or other body surfaces or body cavities exposed during surgery.
16. The method of claim 13, wherein the composition is an oligonucleotide capable of expressing its function inside at least one cell of the mammal.
17. A method of using the composition of claim 1, comprising manufacturing a

medicament for prophylactic or therapeutic treatment of a mammal, the medicament comprising the composition of claim 1.

18. A method of using the composition of claim 1 as a diagnostic agent comprising for use in *in vivo* or *in vitro* diagnostic methods.

19. The use of the composition of claim 17 in the manufacture of a medicament for use in gene therapy, antisense therapy, or genetic vaccination for prophylactic or therapeutic treatment of malignancies, autoimmune diseases, inherited disorders, pathogenic infections and other pathological diseases, comprising introducing the composition into the mammal by administration to

- a) mucosal tissues by pulmonary, nasal, oral, ocular, buccal, sublingual, rectal, or vaginal routes,
- b) submucosal tissues by parenteral routes that are intravenous, intramuscular, intradermal, intracranial, intraspinal, subcutaneous, or intracardiac administration, or
- c) to internal organs, blood vessels, or other body surfaces or cavities exposed during surgery.

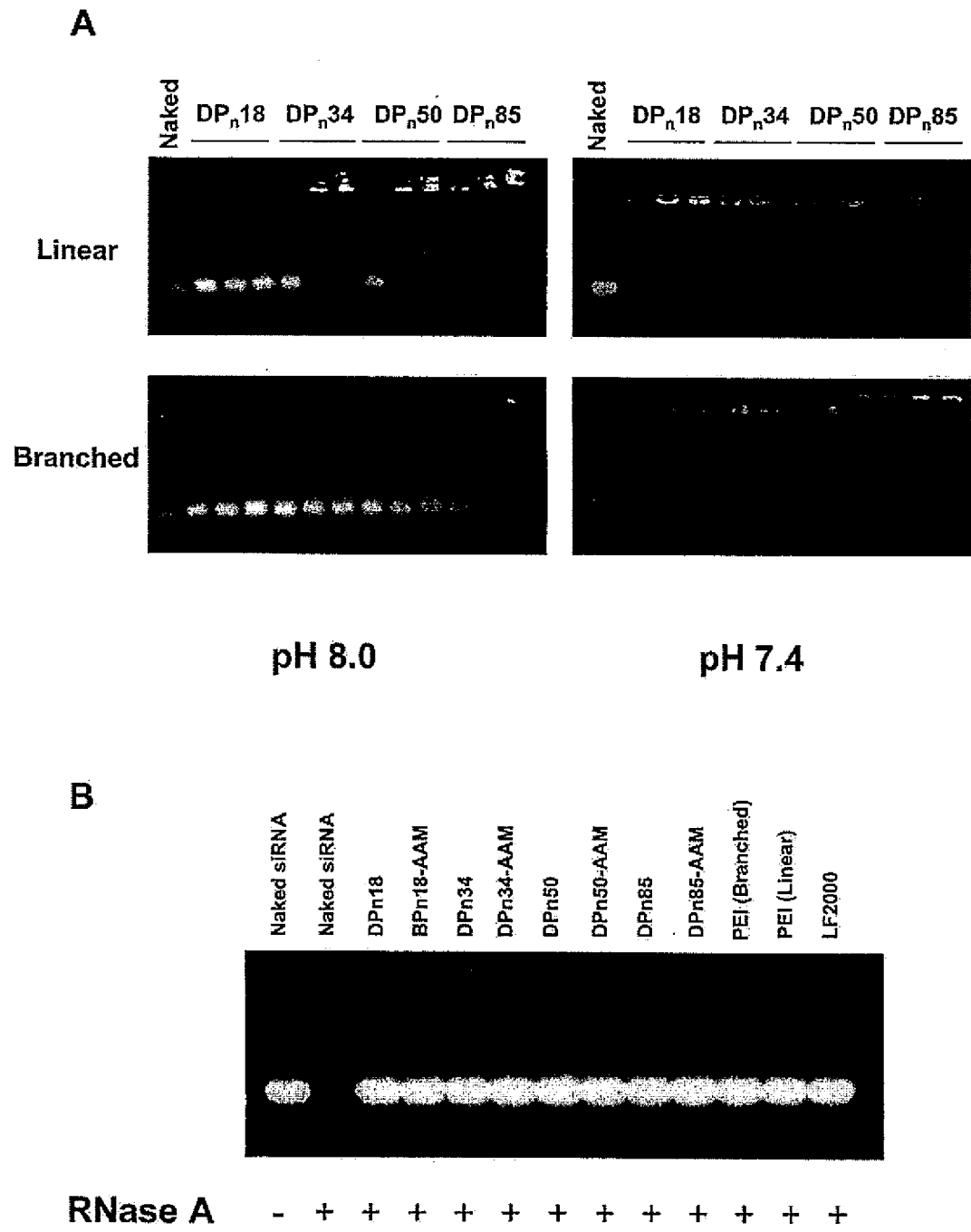


Figure 1

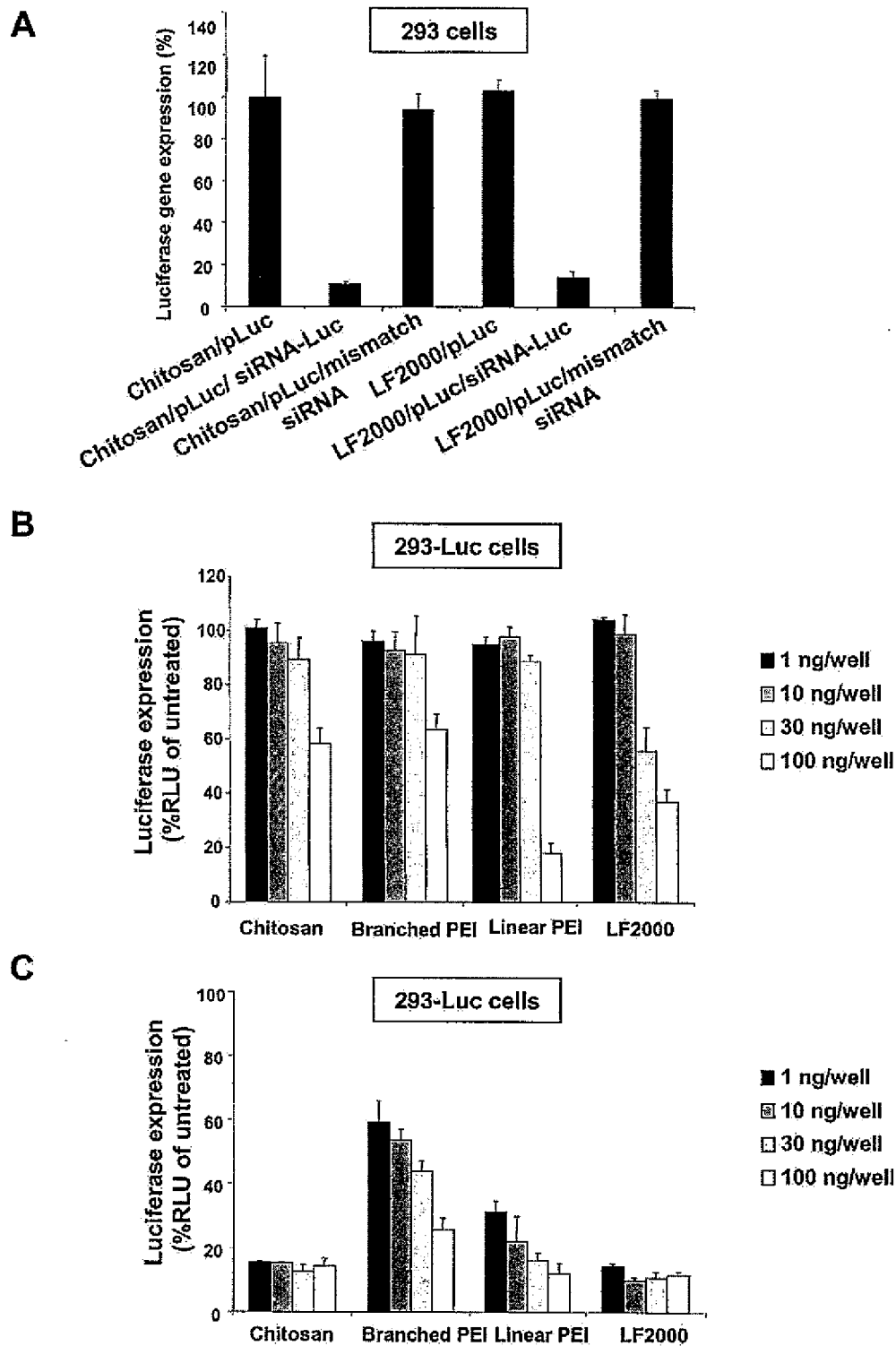
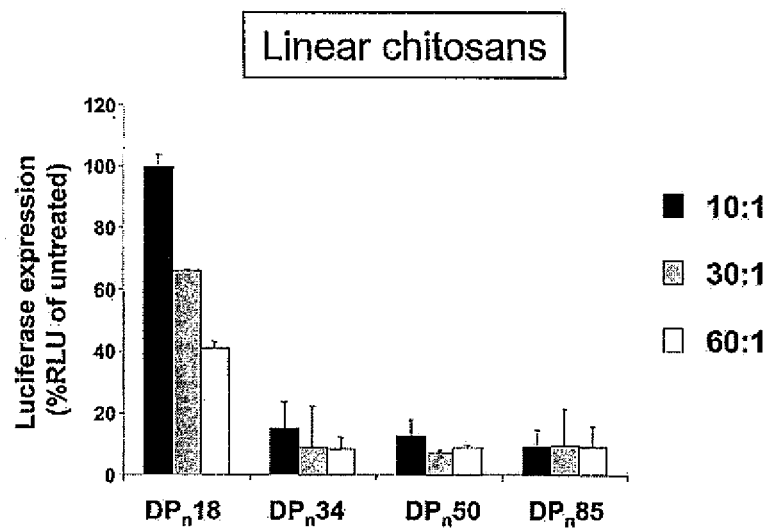
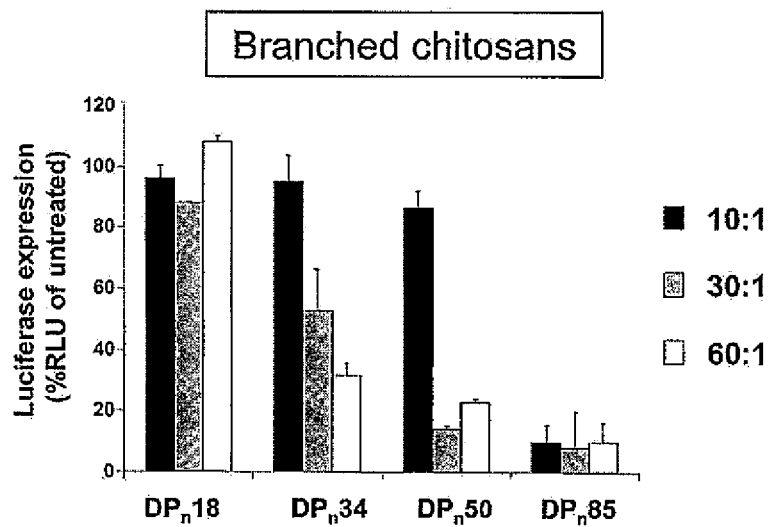


Figure 2

A**B****Figure 3**

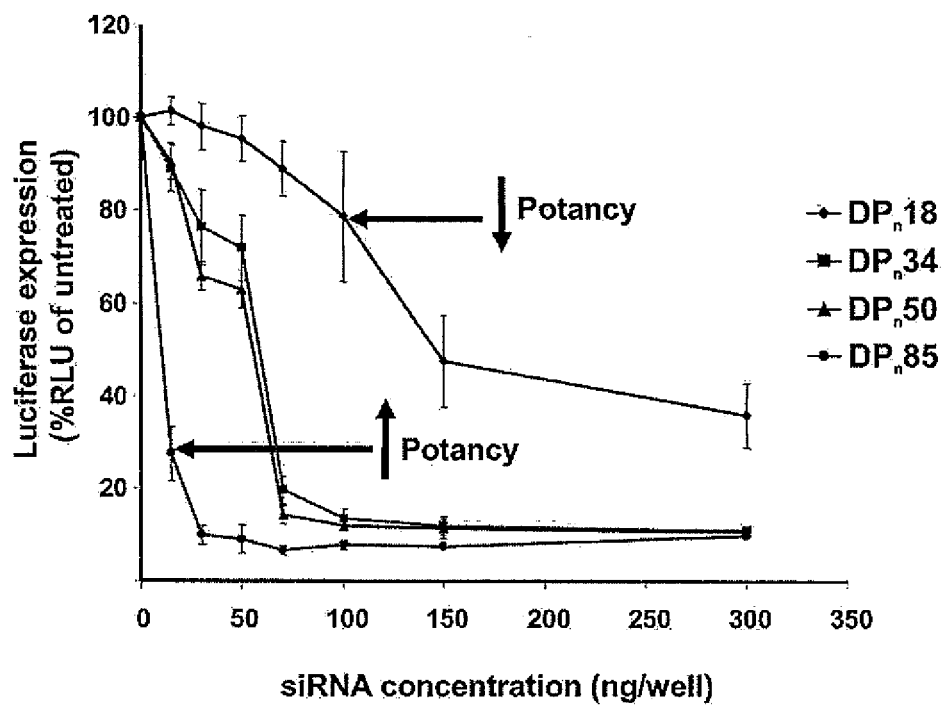


Figure 4

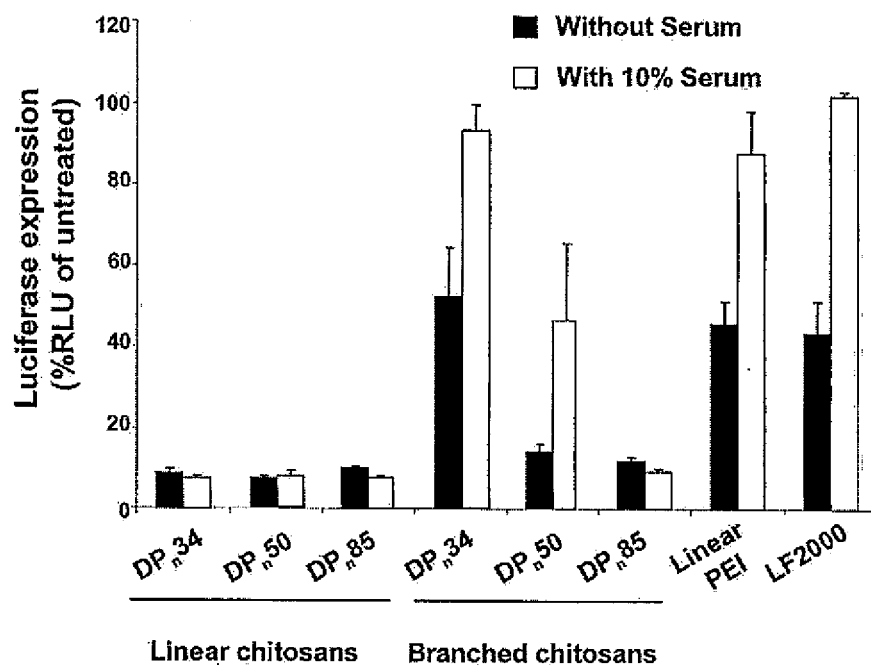


Figure 5

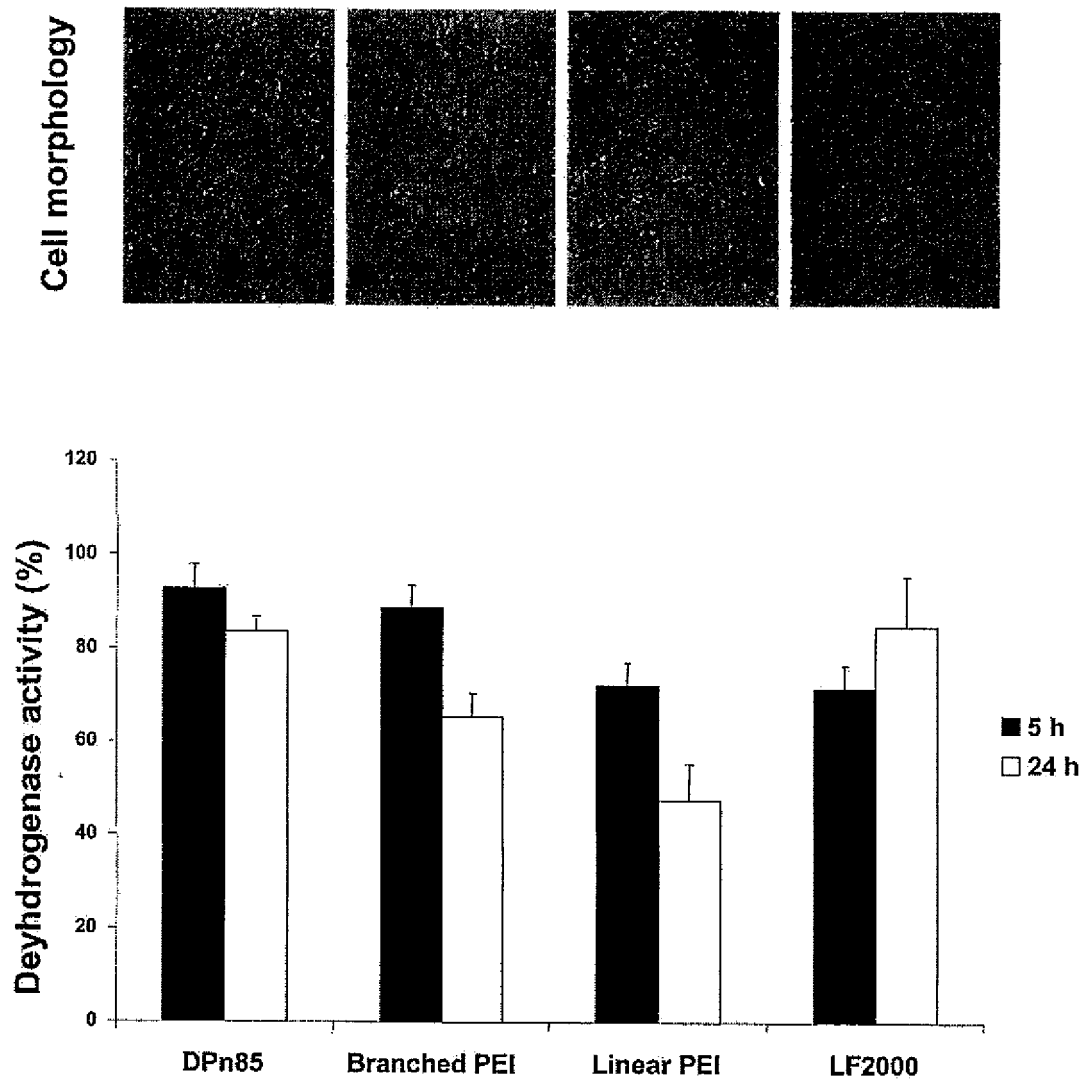


Figure 6

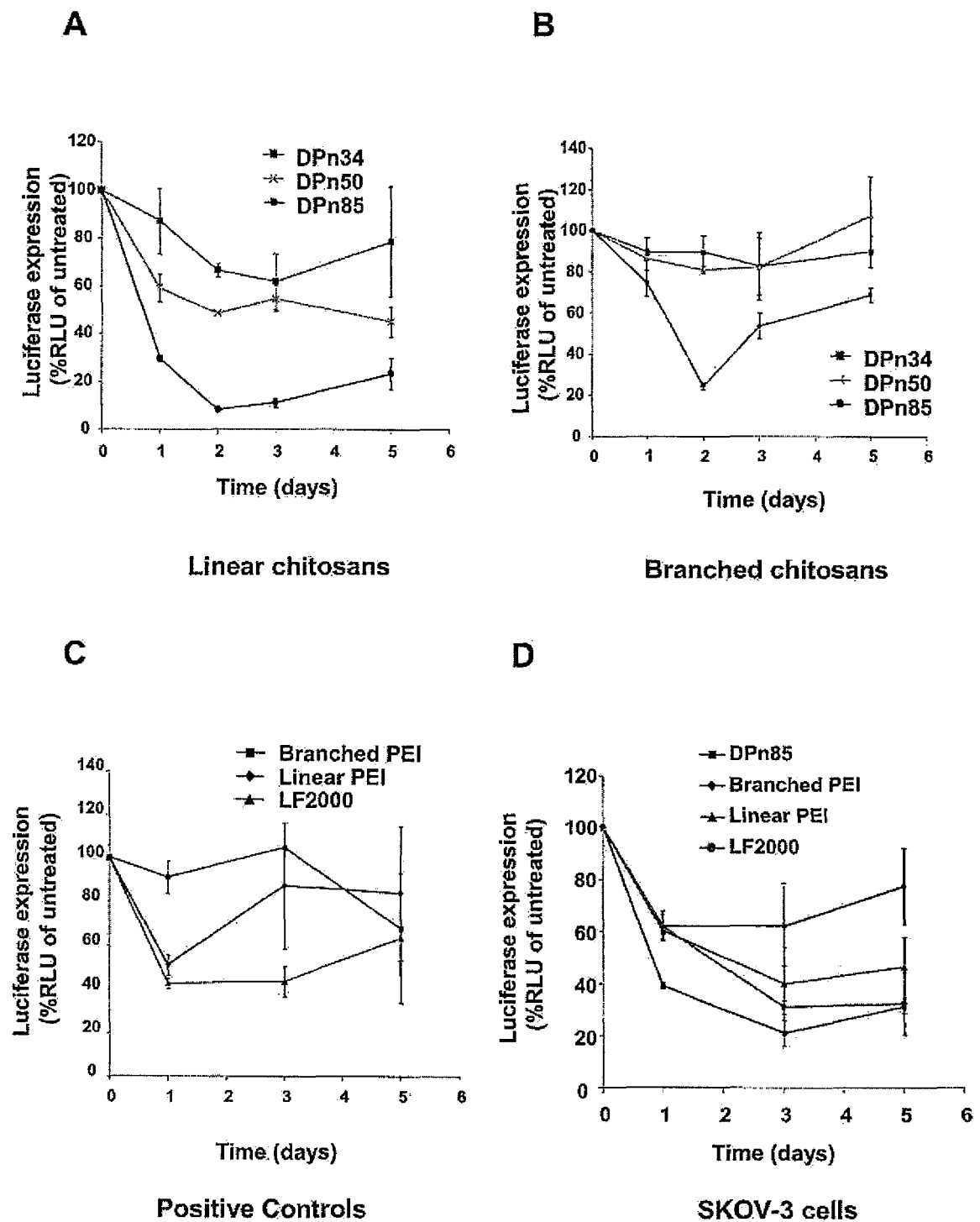


Figure 7