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PEGylated chitosan complexes DNA while improving polyplex colloidal stability and gene transfection efficiency

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ABSTRACT

Chitosan is widely explored as a gene delivery vehicle due to its ability to condense DNA, facilitate transport, and subsequent release allowing gene expression, as well as protecting the DNA. Here, we investigate the enhancement of chitosan–DNA dispersion stability while maintaining transfection efficacy by PEGylation of chitosan. Molecular properties of fully deacetylated chitosans and degree of PEGylation were investigated with respect to compaction of DNA, stability and transfection efficacy. Each of the three chitosan samples with varying chain lengths was PEGylated at three different degrees. The chitosans with degree of PEGylation from 0.6 to 1.9% made polyplexes with DNA. PBS induced colloidal aggregation of polyplexes with initial radius of about 100 nm observed for nonPEGylated chitosans was suppressed for 1.9% PEGylated chitosans. The observed increase in transfection efficacy coinciding with increased polyplex colloidal stability suggests that aggregation of gene-delivery packages may reduce the transfection efficacy.

1. Introduction

Gene transfer using nonviral vectors is a promising approach for safe delivery of nucleic acid therapeutics. DNA delivery faces numerous challenges such as susceptibility to degradation by endogenous enzymes, inefficient cellular uptake as well as poor serum stability and unfavorable pharmacokinetics *in vivo*. Materials used as gene delivery vehicles must support the transport of the plasmids to be expressed and their properties can conceptually be decomposed into various functionalities such as the ability to condense DNA into small sizes to facilitate transport and protect the DNA from endogenous nucleases, facilitate cellular uptake while at the same time also preventing lysosomal degradation, allow intracellular release, and nuclear targeting to allow expression.

One of the polycations intensively investigated for gene transfer is chitosan, a linear binary polysaccharide comprised of (1–4)-linked 2-amino-2-deoxy-β-D-glucose (GlcN) and the *N*-acetylated analogue (GlcNAc). The family of chitosans includes a variety of polymers differing in the fraction of acetylated units (F_A) and molecular weight (M_w), and therefore also different physicochemical and biological properties (Koping-Hoggard et al., 2004; Ma, Lavertu, Winnik, & Buschmann, 2009; Strand, Lelu, Reitan, Davies, Artursson, & Vårum, 2010). It has been shown that DNA compaction, stability of the chitosan–DNA formulation and gene transfer efficiency are critically dependent on both F_A and chain length (Ishii, Okahata, & Sato, 2001; Koping-Hoggard et al., 2004; MacLaughlin et al., 1998; Strand, Danielsen, Christensen, & Vårum, 2005; Strand et al., 2010). It has been recently reported that the chitosans in a narrow interval of chain lengths (DP_n 30–40) were found to be the most efficient gene delivery vehicles *in vitro* (Strand et al., 2005, 2010). Moreover, modification from the common linear chain architecture to branched structures is reported to increase the capacity to enhance transfection of HeLa cells (Malmo, Vårum, & Strand, 2011). Nevertheless, formulations based on generic unmodified chitosans still face several challenges *in vivo*, including poor stability in physiological fluids, aggregation, unwanted interactions with non-target cells and extracellular matrix that eventually may contribute to low transfection efficiency.

To overcome these limitations and improve the functional properties of chitosan carriers, various modifications of chitosans have been introduced. For instance, the substitution of chitosan with different saccharides such as lactose, galactose, and GlcNAc oligosaccharides in order to target lectin receptors on the cell membrane has been reported to also increase the solubility of the chitosans and reduce the aggregation in cell culture media (Issa et al., 2006). PEGylation represent an important approach to affect the solubility of chitosans (Casettari et al., 2012; Germershaus, Mao, Sitterberg, Bakowsky, & Kissel, 2008; Jayakumar et al., 2010; Jiang et al., 2006; Malhotra, Lane, Tomaro-Duchesneau, Saha, & Prakash, 2011; Saranya, Moorthi, Saravanan, Devi, & Selvamurugan, 2011).

The conjugation of poly(ethylene glycol) (PEG) to various biomacromolecules is a well-know strategy to improve their water

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solubility and prolong their blood circulation time (Kitson et al., 1999; Mao et al., 2001; Molineux, 2002; Unezaki et al., 1996). PEGylation has also been shown to increase the solubility of chitosan (Casettari et al., 2012; Saito, Wu, Harris, & Hoffman, 1997). PEGylation of gene transfer carriers is believed to provide a steric shield decreasing the aggregation of complexes and reduce interaction between complexes and plasma proteins, erythrocytes and components of the immune system. For instance, polyplexes prepared with PEG-grafted PEI and PLL were reported to possess longer blood circulation time than those prepared with the unmodified polymers. However, it has also been shown that PEGylation of polymers interfered both with DNA condensation and cell uptake, leading to low transfection efficacy (Merdan et al., 2005; Mishra, Webster & Davis, 2004). In addition to affecting biological activities, grafting of PEG chains on chitosan may also influence the DNA condensation process and the topology of chitosan–DNA polyplexes. It has been reported that chitosans varying in molecular parameters such as F_A and DP form complexes of different topologies (Danielsen, Maurstad, & Stokke, 2005; Danielsen, Vårum, & Stokke, 2004).

It is well established that the efficacy of gene delivery using chitosans is dependent on the unpacking of the polyplexes as determined by the interaction strength between the chitosan carrier and its DNA cargo (Ma et al., 2009). Conjugation of PEG chains to chitosans primarily targeted to increase colloidal stability of chitosan–DNA complexes may influence the interaction with DNA and hence the polyplex stability. The purpose of this study was to develop PEGylated chitosans that combine the advantages of PEGylation to improve colloidal stability, while retaining the DNA condensation ability and gene transfer efficacy of chitosan. This is carried out exploring a range of chain lengths of chitosans, representing various complexation strengths with DNA, and different levels of PEGylation for improving polyplex colloidal stability. Thus, the interplay between chitosan chain length and PEG grafting ratio on the capability for compacting DNA in terms of polyplex size and structure, stability and transfection efficacy, is explored.

2. Materials and methods

2.1. Plasmid DNA

Reporter plasmids gWizLuc (6732 bp) and gWizGFP (5757 bp), containing a cytomegalovirus (CMV) promoter and encoding luciferase and green fluorescent protein, respectively, were purchased from Aldevron (Fargo, ND, USA).

2.2. Chitosans

The characteristics of the chitosan samples are presented in Table 1. The fully de-*N*-acetylated chitosan (F_A < 0.002, M_w 151 kDa) was prepared by heterogenous deacetylation of shrimp chitin in our laboratory. Chitosans with a number average degree of polymerization (DP_n) of 92 and 185 were prepared by nitrous acid depolymerization of chitosan with DP_n 365 followed by $NaBH_4$ reduction (Tommeraas, Strand, Christensen, Smidsrod, & Vårum, 2011). The average degree of polymerization and chain length distributions were analyzed by size-exclusion chromatography (SEC) with refractive index (RI, Dawn Optilab 903, Wyatt Technology) and a multiangle laser light scattering detector (MALLS, Dawn DSP, Wyatt Technology) (Christensen, Vold, & Vårum, 2008). All samples were dissolved in distilled water (MQ water) (5–7 mg/mL) and filtered through 0.22 μm syringe filter (Millipore). The column used was TSK 4000 PWXL, and sample was eluted with 0.2 M ammonium acetate (pH 4.5) at a flow rate of 0.5 mL/min.

2.3. PEGylation

Chitosans were PEGylated by *N*-succinimidyl activated succinyl-PEG (NOF corporation, Japan) with M_w of 5 kDa. For each chitosan, three conjugates with theoretical degrees of substitution (d.s.) of 1%, 2% and 5% of PEG-substituted GlcN were prepared. Freshly prepared PEG solution in MQ-H_2O (50 mg/mL) was added to the solution of chitosan (2 mg/mL) in 50 mM acetate buffer pH 5.5 and the reaction mixture was incubated for 24 h at room temperature. The PEGylated chitosans were extensively dialyzed to remove the unreacted PEG (SpectraPor, cut-off 12–14 kDa). The molecular weight of the conjugates was determined by SEC-MALLS using the same instrumentations and conditions as described above. The samples were applied to elemental analysis (C, N, H) on a Flash Elemental Analyzer 1112 (ThermoQuest, Milan, Italy) to determine the degree of PEGylation of the chitosans. The obtained degree of PEGylation calculated from C/N ratio was similar for all three chain lengths and indicated approximately 30% efficiency of the PEGylation reaction. An overview of the chitosans used in this study is given in Table 1. The chitosan samples are referred to as Chi_x^y, where the numerical values of x and y are the DP_n and degree of PEGylation, respectively.

2.4. Preparation of DNA–chitosan complexes

DNA–chitosan complexes with different amino/phosphate (A/P) ratios and at DNA concentrations of 5–13 μg/mL were prepared by the self-assembly method. Briefly, the required amount of sterile filtered chitosan solution in MQ-H_2O was added to a solution of DNA, and the solution was stirred on a vortex mixer. The complexes were incubated for 30 min at room temperature before characterization or further use.

2.5. Size and zeta potential measurements

The size of the DNA–chitosan complexes was determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, UK). A minimum of two independent samples were measured, with three replicates for each sample. The *z*-average sizes of the complexes were obtained by cumulant analysis of the correlation function using the viscosity and refractive index of water in the calculations, and with the particles modelled as spheres.

For measurements of aggregation kinetics, the solution of complexes prepared at C_{DNA} = 13.3 μg/mL in MQ-H_2O was diluted 1:2 in double-strength phosphate-buffered saline (PBS), pH 7.2. In this way, isotonic formulations similar to transfection experiments

Table 1
Parameters of the chitosan samples, all with F_A < 0.002. The molecular weights (M_w, M_n) and polydispersity index (PDI) were determined by SEC-MALLS. The obtained degree of PEGylation was calculated from C/N analysis of the prepared samples.

Chitosan sample	DP_n	PEG degree	M_w	M_n	PDI
Chi_{92}^{0}	92	0%	31 600	18 400	1.72
$Chi_{92}^{0.7}$		0.67	36 900	25 000	1.48
$Chi_{92}^{0.9}$		0.91	38 900	26 700	1.46
$Chi_{92}^{1.9}$		1.89	45 400	30 900	1.47
Chi_{189}^{0}	189	0%	75 700	37 700	2.00
$Chi_{189}^{0.7}$		0.69	82 400	44 700	1.84
$Chi_{189}^{1.0}$		0.96	85 600	47 000	1.82
$Chi_{189}^{1.9}$		1.89	98 500	54 200	1.82
Chi_{365}^{0}	365	0%	151 000	73 000	2.07
$Chi_{365}^{0.6}$		0.62	167 000	84 400	1.98
$Chi_{365}^{1.0}$		0.99	176 000	86 700	2.07
$Chi_{365}^{1.9}$		1.94	202 000	100 000	2.02

were obtained. The size was then measured every 8th minute for 140 min.

The zeta potential, ζ, of the complexes was determined by laser Doppler velocimetry in combination with phase analysis light scattering (Zetasizer Nano ZS). The zeta potentials were determined for complexes prepared in 5 mM NaCl at C_{DNA} = 6.65–10 μg/mL.

2.6. Atomic force microscopy imaging

For AFM imaging, DNA–chitosan complexes were prepared at C_{DNA} = 6.65 μg/mL, A/P 10 in MQ-H_2O. A 10 μL aliquot of the sample was deposited on a freshly cleaved mica disk (mineral muscovite, SPI Supplies), incubated ~2 min, before being dried in a stream of N_2 followed by vacuum drying for 2 h or more at 1.3×10^{-4} Pa. The samples were imaged by tapping mode AFM (Digital Instruments Multimode IIIa) in air as described previously (Maurstad, Danielsen, & Stokke, 2003; Stokke, Falch, & Dentini, 2001), employing cantilevers with a nominal resonance frequency of 330 kHz and nominal spring constant of 42 N/m (nanosensors). Multiple AFM topographs, typically 10–15, were obtained as basis for the assessment of the type of geometries of the polyplexes.

2.7. EtBr fluorescence exclusion assay: compaction process and serum stability

EtBr (Sigma Aldrich, stock solution of 1 mg/mL) was added to the DNA solution (C_{DNA} = 5 μg/mL) to a ratio of one EtBr molecule per 12th base pair of DNA. The fluorescence was measured using a Spex Spectramate Fluorolog (Spex Industries, Inc., Metuchen, NJ), with excitation wavelength 511 nm and recording the emission spectrum up to 700 nm. A measure of the emitted fluorescence intensity was obtained from the spectrum by averaging the intensity within the interval 595–610 nm. The complexation of DNA by the different chitosans was investigated in 5 mM acetate buffer pH 5.5 by stepwise addition of chitosan at time intervals ~15 min, up to an A/P-ratio of 10. The results are averaged for two independent measurements. Furthermore, the stability of the complexes following addition of serum was investigated. For the stability measurements, complexes were prepared at an A/P ratio of 10 in 5 mM acetate buffer, pH 5.5 and in MQ-H_2O. EtBr was added to the solution and the fluorescence intensity measured for ~2 h and using the fluorescence intensity before adding serum as the reference.

Normalized titration curves were reported, where the fluorescence intensity was normalized relative to the fluorescence intensity of DNA–EtBr before addition of chitosan for the titration, and relative to the fluorescence of the solution of complexes and EtBr before addition of serum for the stability testing. As a control for the stability experiment, serum was added to a DNA–EtBr solution (without chitosan) and the fluorescence signal determined as for the complexes.

2.8. Agarose gel electrophoresis

The DNA retention capacity of the polyplexes and serum induced release of DNA was studied using gel electrophoresis performed on 0.8% agarose gel in 40 mM Tris–acetate–EDTA (TAE) buffer at pH 8.0. The polyplexes were prepared as described above at a constant DNA concentration of 13 μg/mL and A/P ratios of 5, 10, 30 and incubated for 30 min. Next, the polyplexes were diluted 1:2 by transfection medium with and without 10% FBS and incubated at 37 °C, 5% CO_2 for 4 h. After incubation, 10 μL of the complex solution was mixed with 5 μL of loading buffer and loaded into the wells. The electrophoresis was run at 80 V for 45 min and the DNA was stained by EtBr (0.5 μg/mL).

2.9. In vitro transfection experiments

Human embryonic kidney cell line HEK293 was obtained from ATCC (Rockville, MD) and cells between passage numbers 39 and 60 were used in all experiments. The HEK293 were grown in MEM (Gibco Invitrogen) supplemented with 1 mM of non-essential amino acids and 10% fetal bovine serum (FBS, Gibco Invitrogen) at 37 °C and 5% CO_2.

The HEK293 were seeded in 96-well tissue culture plates (Corning Cell-bind #3300) and grown to the cell confluency of 80–90% on the day of transfection. The polyplexes formulated in MQ water were diluted 1:2 by OptiMEM (OptiMEM I, Gibco Invitrogen) supplemented by 270 mM mannitol and 20 mM HEPES to adjust osmomolality and pH of the formulation. Cells were washed with pre-heated OptiMEM, and 50 μL of isotonic formulation containing 0.33 μg pDNA was added to each well. After 5 h of incubation, the medium including the polyplexes was replaced by 200 μL of fresh culture medium (MEM, 10% FBS) and cells were further incubated for 48 h. To determine luciferase expression, cells were washed and lysed with luciferase lysis buffer (Promega, Madison, USA) and the luciferase activity (RLU) was measured on a luminometer (Molecular Devices, USA). The total cell protein was determined by bicinchoninic acid assay (Pierce).

3. Results

3.1. Physicochemical characterization of complexes

The complexation of DNA by chitosan was monitored based on the concomitant displacement of EtBr from the intercalating position of the DNA helix with the largest increase in fluorescence quantum efficiency thus decreasing of fluorescence intensity. The EtBr fluorescence following titration of EtBr–DNA with different chitosans shows that all chitosans induce similar reduction of fluorescence intensity when an A/P ratio of 2–3 is reached (Fig. 1 for titration curves for chitosans in the series Chi_{92} and Chi_{365}). The plateau value reached in the titration curves is in the range 0.24–0.32 for all the chitosans, showing that they are able to compact DNA to similar extents. There is no apparent systematic difference in the slope of the titration curves during the first part (A/P <3) of the titration between chitosans of different degrees of PEGylation.

Measurements of the *z*-average diameter of the polyplexes by dynamic light scattering, revealed no large differences for the different chitosans to compact DNA at the three different A/P ratios (Table 2). The *z*-average diameters of the DNA–chitosan complexes were observed to be less than 100 nm for most of the samples. The

Table 2
z-Average diameter and zeta potential of the complexes formed when using chitosans of different DP and different degree of PEGylation to compact DNA.

Chitosan	z-average diameter (nm)			Zeta potential (mV)		
	A/P 5	A/P 10	A/P 30	A/P 5	A/P 10	A/P 30
Chi_{92}^{0}	78 ± 4	109 ± 5	72 ± 3	32 ± 2	33 ± 1	38 ± 1
$Chi_{92}^{0.7}$	81 ± 8	101 ± 6	75 ± 1	18 ± 2	11 ± 3	11 ± 2
$Chi_{92}^{0.9}$	76 ± 5	114 ± 10	86 ± 9	15 ± 1	13 ± 2	12 ± 5
$Chi_{92}^{1.9}$	75 ± 2	87 ± 14	81 ± 5	13 ± 4	11 ± 1	16 ± 2
Chi_{189}^{0}	72 ± 6	63 ± 1	94 ± 13	34 ± 3	31 ± 2	31 ± 3
$Chi_{189}^{0.7}$	80 ± 1	93 ± 20	92 ± 11	19 ± 2	16 ± 6	24 ± 2
$Chi_{189}^{1.0}$	73 ± 2	103 ± 24	84 ± 3	15 ± 4	19 ± 3	25 ± 3
$Chi_{189}^{1.9}$	76 ± 2	98 ± 3	120 ± 35	16 ± 2	16 ± 2	22 ± 5
Chi_{365}^{0}	79 ± 7	67 ± 6	125 ± 12	30 ± 3	34 ± 1	31 ± 3
$Chi_{365}^{0.6}$	82 ± 8	89 ± 9	113 ± 5	21 ± 4	26 ± 7	17 ± 3
$Chi_{365}^{1.0}$	82 ± 8	98 ± 7	139 ± 43	17 ± 2	10 ± 4	24 ± 3
$Chi_{365}^{1.9}$	91 ± 3	168 ± 3	151 ± 25	12 ± 1	11 ± 2	25 ± 2

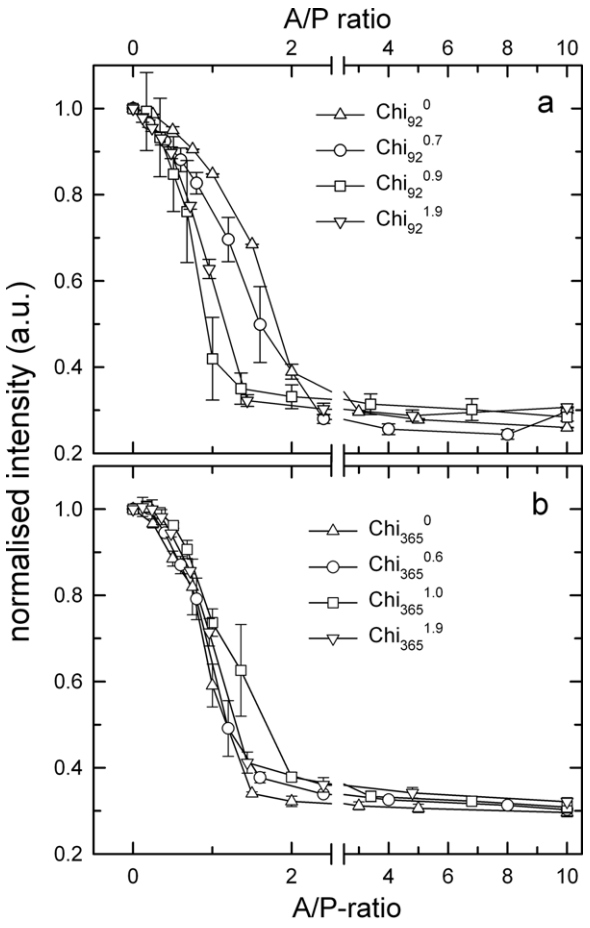

Fig. 1. Relative EtBr fluorescence emission intensity of DNA–EtBr *versus* concentration of chitosan added to the aqueous solution of DNA–EtBr. The data monitor effects of complex formation of DNA using chitosans with various degrees of PEGylation and for two different degrees of polymerization (a) Chi_{92} series and (b) Chi_{365} series.

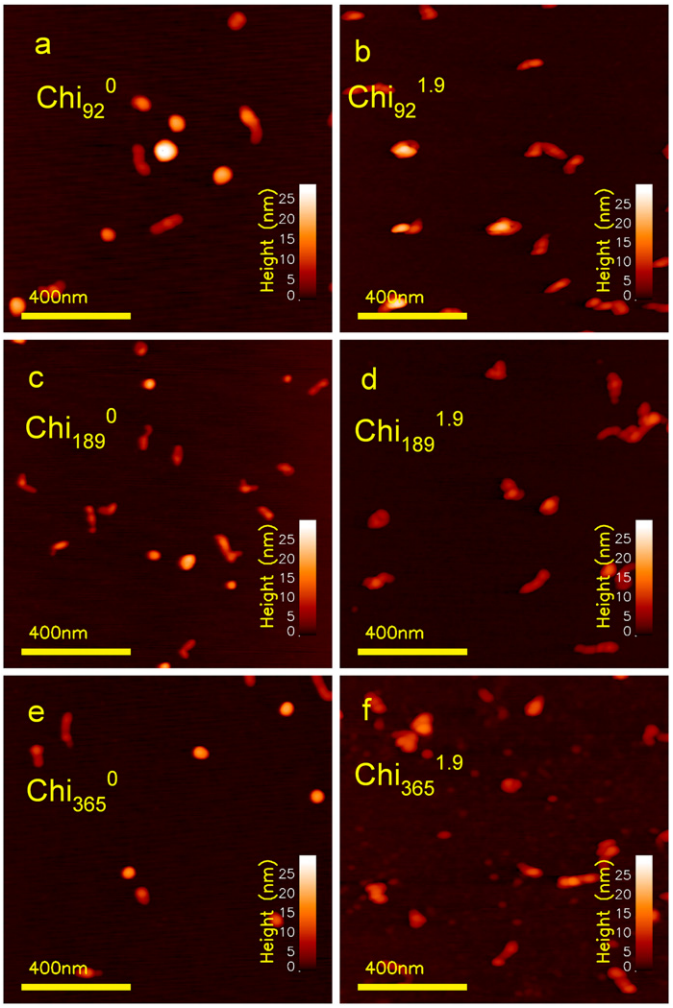

Fig. 2. Tapping mode AFM topographs of DNA–chitosan complexes employing chitosans of different degree of polymerization and with different degree of PEGylation. Selected AFM topographs are shown for increasing molecular weight of the nonPEGylated chitosans (a, c and e) and the 1.9% PEGylated chitosans (b, d and f) as indicated. The chitosans–DNA complexes were all prepared in distilled water at an A/P-ratio of 10.

exception is Chi_{365}, where all four chitosan samples (nonPEGylated and PEGylated) at an A/P-ratio of 30 resulted in complexes with a *z*-average diameter larger than 100 nm. This was also the case for $Chi_{365}^{1.9}$ at an A/P ratio of 10 and $Chi_{189}^{1.9}$ A/P-ratio of 30.

The zeta potential, ζ, of the complexes was determined in 5 mM NaCl. Most of the data gave a value of ζ in the range 30–34 mV for the complexes prepared using DNA and the unmodified chitosan, with no change in ζ for increasing A/P ratio. The value of ζ was also independent on the chain length of the unmodified chitosans. Complexes of the PEGylated chitosan and DNA displayed a reduced, but still positive, zeta potential (Table 2). A tendency to yield a less positive ζ when employing the PEGylated chitosans was found for the three average chain lengths of chitosan and A/P-ratios 5, 10 and 30. Generally, increasing the degree of PEGylation from 0.7% to 1.9% did not lead to significantly lower zeta potentials.

DNA–chitosan polyplexes adopt different structures; toroids, rods and globules (Danielsen et al., 2004), with fraction of different structures depending on molecular properties of the chitosan and solution conditions. The complexes were therefore characterized by tapping mode AFM. The complexes yielded similar type of morphologies, but with relative abundance and other details depending both on the chitosan DP and for each DP, the degree of PEGylation (Fig. 2). DNA complexes prepared using the nonPEGylated chitosans at various chain lengths yielded linear and globular structures. Toroids were not observed to constitute a substantial fraction of the morphologies in any of the samples. Generally, an increase in the degree of PEGylation resulted in less defined structures.

3.2. Stability of complexes

The apparent stability of the chitosan–DNA complexes (A/P 10) was determined based on the recovery of EtBr fluorescence intensity after addition of serum to the solutions (Fig. 3). For complexes prepared in 5 mM acetate buffer pH 5.5, PEGylation of chitosans yielded more stable complexes than nonPEGylated chitosans using this assay (Fig. 3). However, when the complexes were prepared in MQ-H_2O, the stability of the complexes when prepared with PEGylated chitosans as compared to nonPEGylated chitosan varied with the chain length of the chitosan, giving rise to a less straightforward pattern. The preparation of polyplexes in aqueous solution without added salt prior to being used for transfection studies are common in *in vitro* studies (Koping-Hoggard et al., 2004). The destabilization of the chitosan–DNA complexes after adding serum was a rapid process with most of the change occurring during the first few minutes and the change in the relative fluorescence intensity levelled off within 10 min after introducing serum.

Gel electrophoresis assay showed that all chitosans formed stable complexes at A/P ratios of 10, 30 and 60 that did not release DNA following incubation in the transfection medium with or

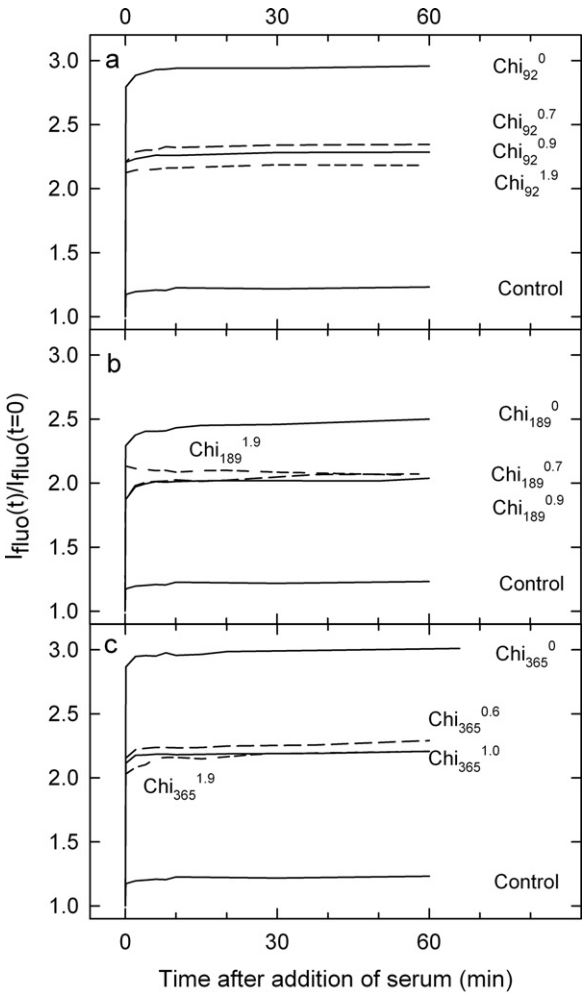

Fig. 3. Stability of complexes when serum is added, measured by the recovery of EtBr fluorescence. The complexes were prepared in distilled water at an A/P ratio of 10, and the control is DNA–EtBr exposed to serum. (a) Chi_{92}; (b) Chi_{189} and (c) Chi_{365}.

without serum. The lack of electrophoretic bands corresponding to naked DNA in medium with serum indicated that degradation of DNA is not prevalent under our conditions. Thus, all chitosans efficiently protected complexed DNA from eventual degradation by nucleases.

The colloidal stability of the complexes was investigated by determination of the hydrodynamic size following addition of PBS after preparing the complexes initially in distilled water. The stability was investigated for all chitosans at A/P ratios 5, 10 and 30. During the first 20 min after addition of PBS, a rapid increase in size was observed, followed by a slower growth leveling off within the time frame studied here (2.5 h). The chitosans with the highest degree of PEGylation (1.9%) formed the DNA complexes that were least susceptible to further aggregation (Fig. 4). However, it is only at an A/P ratio of 5 for all three chain lengths of chitosan and Chi_{92} and Chi_{185} at A/P 10 that complexes formed between DNA and PEGylated chitosans do not aggregate to any large extent. The colloidal stability for the 1.9% PEGylated chitosans was observed to decline with increasing A/P ratio beyond 10 (Fig. 4). Complexes between DNA and $Chi_{365}^{1.9}$ increased their mean size, but remained below μm within the investigated time frame. For all other conditions and chitosans, the complexes aggregate to μm sizes upon addition of PBS.

3.3. Transfection efficacy

The transfection efficacy using the PEGylated-chitosan as delivery vehicles depended on the chain length of chitosan, PEGylation degree and the A/P ratio of the preparation. The gene transfection efficacy decreased substantially with increasing chain length of chitosan for all PEGylation degrees (Fig. 5). The transfection efficacy of Chi_{92} was approximately 3–5 and 50–100 folds higher than for Chi_{185} and Chi_{365}, respectively. Comparing the effect of PEGylation, 1% and 1.9% PEGylated chitosans mediated higher gene transfer efficacy than the nonPEGylated biopolymers. Furthermore, for non-PEGylated, 0.7% PEGylated and partly also 1% PEGylated chitosans, the efficacy decreases rapidly with increasing A/P ratio. For the 1.9% PEGylated samples, the transfection efficacy became less sensitive to A/P ratio. Comparing all chitosans at the three tested A/P ratios, the shortest chitosan at a PEGylation degree of 0.9% ($Chi_{92}^{0.9}$), A/P ratio 5 and 10, and at PEGylation degree of 1.9% ($Chi_{92}^{1.9}$) at all A/P ratios, gave the highest, and comparable, transfection efficacies. Although the chain length of the employed PEG may affect a charge driven polyplex formation capability larger than the degree of substitution, we cannot from the results presented herein exclude the possibility that a degree of PEGylation larger than 1.9% may yield more efficient transfection.

4. Discussion

Addressing challenges in design of successful gene delivery vehicles include simultaneously fulfilling abilities to transport, protect, localize and at the same time maintain the released nucleotides to be transcribed. It is therefore imperative to perform characterization of various properties of the resulting complexes and how these are affected by modification of key properties of the gene delivery vehicles. Thus, we performed PEGylation at three different degrees of substitution for three different chain lengths of chitosan followed by both relevant physicochemical characterization and *in vitro* transfection assay to monitor various properties.

The observed decrease in a positive zeta potential associated with the PEGylation (Table 2), also at the lowest degree of substitution of PEG is analogous to that reported for a triblock polymer with PEGylated segments flanking a cationic core compared to the polycation (Rackstraw, Stolnik, Davis, Bignotti, & Garnett, 2002). Their suggestion that the PEG chain displaces the fluid dynamic shear plane further away from the polyplex core and thereby reduce the zeta potential, appears a valid interpretation also for chitosan. Based on differences in chain lengths, the molecular ratio of chitosan-DNA is likely larger than 1, which is in support of a hydrophilic corona consisting of several PEG groups.

The fluorescence quantum yield of ethidium bromide (EtBr) increases when intercalated in the DNA helix (Boger, Fink, Brunette, Tse, & Hedrick, 2001; Kim, Yamasaki, Jang, & Kataoka, 2010). Condensation of DNA or destabilization of DNA complexes affect EtBr quantum yield resulting in decrease or increase, respectively, of fluorescence emission. The data show that both unmodified and PEGylated chitosan are able to compact the pDNA for all degrees of substitution and that there is no further change in the EtBr exclusion assay beyond an A/P ratio larger than 2 (Fig. 1). All the chitosans with degrees of PEGylation up to 1.9% were able to effectively displace the dye (Fig. 1), similar to that for partially deacetylated chitosans with a F_A of 0–0.15 (Strand et al., 2005). The AFM imaging shows that apparent globular and extended structures are most abundant, whereas toroids are not preferred. This is in line with previous reports of chitosan induced compaction of DNA where toroids have been a less abundant geometry for complexes prepared using rather high molecular weight DNA (Danielsen et al.,

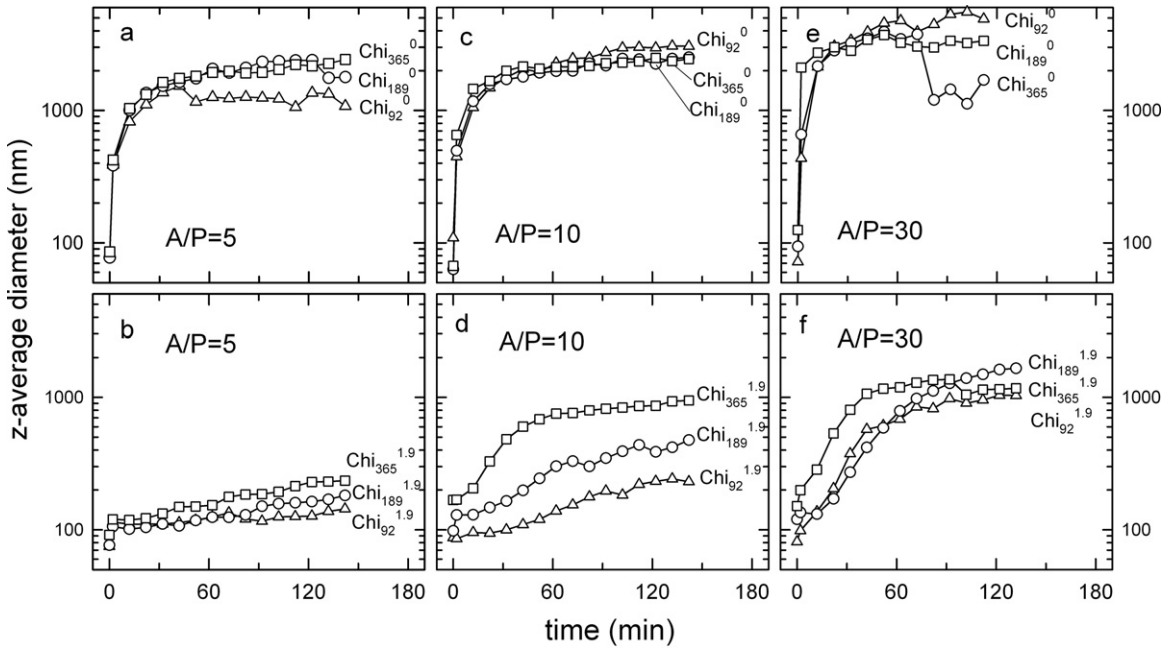

Fig. 4. Hydrodynamic radius of chitosan–DNA complexes *versus* time after adding phosphate buffered saline (PBS) to the complexes initially prepared in distilled water and at amino to phosphate ratio (A/P ratio) of 5 (a and b), A/P = 10 (c and d) and A/P = 30 (e and f). The change in solvent conditions by PBS was induced for chitosan–DNA complexes prepared using either unmodified chitosan samples Chi_x^0, $x = 92$, 189 or 365, where parameter x denote the number average degree of polymerization (a, c and e) or PEGylated chitosans with degree of substitution 1.9% chitosan samples $Chi_x^{1.9}$, $x = 92$, 189 or 365 (b, d and f).

2005). Although introduction of PEG on chitosan potentially can interfere with the electrostatic driven complexation, as suggested in a study by Rackstraw et al., where toroidal condensates were suppressed by PEG groups (Rackstraw et al., 2001), our data do not support a major influence on the overall ability for complexation for the degrees of PEGylation up to 1.9% (Figs. 1 and 2). Thus, the interaction of PEGylated chitosans with DNA is sufficiently strong to form polyplexes for chitosans with degree of PEGylation up to 1.9%. This is similar to the thermodynamic analysis of DNA condensation induced by PEG-block PLL polyions that reported to maintain an entropic driven first binding stage that is a signature of an polyanion–polycation mechanism (Kim et al., 2010).

When used to monitor effects of adding serum to the solution the EtBr assay do reveal differences (Fig. 3). While chitosan–DNA complexes prepared using the non-PEGylated chitosan consistently show the largest increase in the EtBr fluorescence emission intensity on addition of serum for all chain lengths of chitosan, the complexes prepared using the PEGylated chitosan reveal a smaller increase in the relative EtBr fluorescence. At the same time, the gel electrophoresis assay does not indicate release of DNA on exposure to serum. These observations are interpreted in view of differences in the distribution of binding modes of the EtBr (Boger et al., 2001; Piosik, Wasielewski, Woziwodzka, Sledz, & Gwizdek-Wisniewska, 2010; Tse & Boger, 2004). The present observations lead to the suggestion that serum components interact with the chitosan–DNA complexes and displace EtBr from binding sites with the largest increase in quantum yield due to its intercalation between the DNA basepairs.

PEGylated polycations have been suggested to form complexes with DNA where the electrostatic interactions between the cationic segments and DNA are maintained while the PEG segments forms a hydrophilic corona surrounding the particle cores (Rackstraw et al., 2001). The present observation of the colloidal stability of complexes using the PEGylated chitosan and DNA do show a substantial effect of PEGylation of the chitosan on the growth of the hydrodynamic diameter with time (Fig. 5), with the A/P-ratio also influencing the stability. Reduction of the zeta potential and increase of the separation between the complexes due to a hydrophilic corona and changes in these due to electrostatic screening, are possible molecular mechanisms involved in suppression the cluster growth. Nevertheless, the finding for complexes prepared at A/P = 10 using the 1.9% PEGylated chitosan, indicate that consideration of zeta potentials only (Table 2) is not sufficient to account for the observation. At A/P = 10 it is suggested that some of the excess chitosan are not included in the complexes as reported (Thibault et al., 2011), and that such excess chitosan may

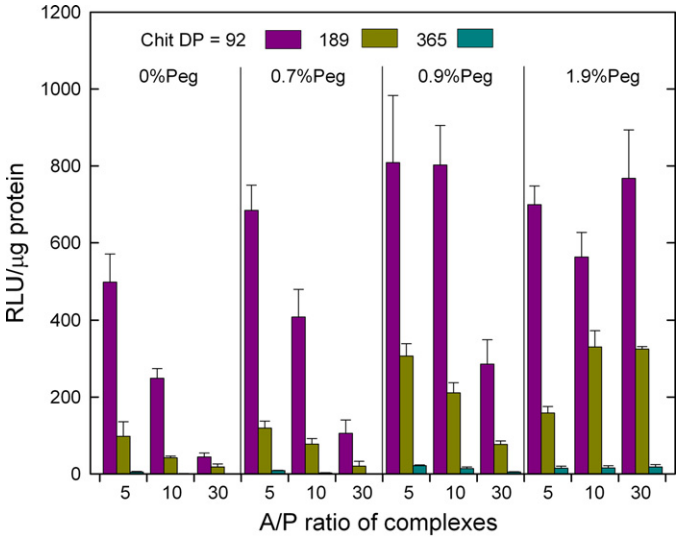

Fig. 5. Luciferase gene expression in HEK293 cells transfected with chitosans and PEGylated chitosans at constant total 0.33 mg pDNA (plasmid gWizLuc) per well and using chitosan amine to DNA phosphate (A/P) ratio 5, 10 and 30. Chitosan samples with three different degree of polymerization and each modified to three different degrees of PEGylation were used. The relative luciferase gene expression is shown as the average of four experiments with standard deviation.

contribute to the observed colloidal instability through *e.g.* a bridging mechanism.

The *in vitro* transfection of the HEK293 cells using the luciferase reporter gene indicates that the highest transfection efficiency was obtained for Chi_{92}, and that increasing the chain length consistently reduced the transfection efficiency. For the non- and 0.7% PEGgylated chitosans, a constant decline in transfection efficiency was observed with increasing A/P ratio, whereas the trends were less evident for the more PEGylated chitosans. The increased transfection efficiency with increasing PEGylation, *e.g.* the most effective chitosan being $Chi_{92}^{0.9-1.9}$ at A/P = 5 coincided with conditions where the complexes were found to have the largest colloidal stability, and therefore can be translocated across the membrane as the smallest entities. The observed reduction in transfection for A/P ratio beyond 10 (except for $Chi_{92}^{1.9}$) coincides with polyplex compositions prone to colloidal aggregation on transfer to PBS. The transfection data also revealed that the increase in transfection with increasing degree of PEGylation is shifted to a larger degree of PEGylation for the highest molecular weight chitosan. This indicates that similar destabilization effects of polyplexes using PEGylation of chitsoan need larger degree of PEGylation the higher the molecular weight of chitosan. Erbacher and coworkers suggested that the increase of transfection using gluconoylated poly-L-lysine as a gene delivery vehicle originated from decreasing interaction strengths between PLL and DNA (Erbacher, Roche, Monsigny, & Midoux, 1997). Both polyplex unpacking during cell internalization and subsequent accessibility of DNA for transcription are among the critical steps for gene delivery systems (Strand et al., 2005). An intermediate stability of the polyplexes is one of the criteria that need to be met for a successful gene delivery vehicle. The EtBr displacement assay does indicate that PEGylation perturb packing modes of the polyplexes, and the observed increase in transfection can therefore arise due to such a PEG-induced decreased stability of the polyplex.

5. Conclusions

The physico-chemical properties and gene delivery ability of DNA complexes formed with PEGylated chitosan depend both on the degree of PEGylation and chain length of the chitosan. Complexes formed with 1.9% PEGylated chitosan remained stable in PBS and showed increased transfection efficacy compared to nonPEGylated chitosans.

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