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Research paper

Gene silencing activity of siRNA polyplexes based on biodegradable polymers

Amir K. Varkouhi [a], Twan Lammers [a,b], Raymond M. Schiffelers [a], Mies J. van Steenbergen [a], Wim E. Hennink [a], Gert Storm [a,*]

[a] Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands
[b] Department of Experimental Molecular Imaging, RWTH – Aachen University, Aachen, Germany

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ABSTRACT

Cationic polymers are used as non-viral vectors for nucleic acid delivery. In this study, two biodegradable cationic polymers were evaluated for the purpose of siRNA delivery: pHPMA-MPPM (poly((2-hydroxypropyl) methacrylamide 1-methyl-2-piperidine methanol)) and TMC (O-methyl-free N,N,N-trimethylated chitosan). The silencing activity and the cellular cytotoxicity of polyplexes based on these biodegradable polymers were compared with those based on non-biodegradable pDMAEMA (poly(2-dimethylamino)ethyl methacrylate) and PEI (polyethylenimine) and with the regularly used lipidic transfection agent Lipofectamine. To promote endosomal escape, either the endosomolytic peptide diINF-7 was added to the formulations or photochemical internalization (PCI) was applied. Incubation of H1299 human lung cancer cells expressing firefly luciferase with polyplexes based on pHPMA-MPPM and TMC showed 30–40% silencing efficiency. This silencing activity was equal to or better than that obtained with the standard transfectants. Under all experimental conditions tested, the cytotoxicity of the biodegradable polymers was low. The application of PCI, as well as the addition of the diINF-7 peptide to the formulations increased their silencing activity up to 70–80%. This demonstrates that pHPMA-MPPM- and TMC-based polyplexes benefit substantially from endosomal escape enhancement. Importantly, the polyplexes retained their silencing activity in the presence of serum, and they showed low cytotoxicity. These biodegradable vectors are therefore attractive systems for further in vivo evaluations.

1. Introduction

Since the first description of RNA interference (RNAi), considerable research efforts have been dedicated to use small interfering RNAs (siRNA) as novel biotherapeutics [1]. These double-stranded RNA molecules are able to silence genes effectively in a sequence specific manner [2,3]. However, siRNA has unfavorable biopharmaceutical properties. They are rapidly degraded by nucleases present in biological fluids and have low membrane penetration capabilities and therefore do not reach in sufficient amounts the cellular cytoplasm, where the RNA interference (RNAi) machinery is located [4,5]. Consequently, a key challenge to the effective and widespread use of this new class of biotherapeutics is their cytosolic delivery [6].

Cationic polymers have been studied for nucleic acid delivery in the last decade. Due to their positive charge, these polymers are able to complex with anionic nucleic acids to form polyplexes [7]. However, many of these cationic polymers show considerable toxicity both towards in vitro cultured cells and in animal models. The toxicity is attributed to their cationic character, and, additionally, to the fact that many of the studied polymeric gene/siRNA vectors are non-degradable. Therefore, the search for effective but non-toxic vectors is currently of prime interest [8]. It has been shown that biodegradable polymers are attractive candidates for the design of siRNA-loaded polyplexes [9,10]. Our group has reported on several biodegradable polymers which have been evaluated for DNA delivery, both in vitro and in vivo. One example is pHPMA-MPPM (Fig. 1). This polymer contains a biodegradable linker which is stable at pH 5 (pH in endo/lysosome) and is degraded at pH 7. The colloidal stabililty of polyplexes based on this polymer was investigated by Luten et al. (2006). It was shown that the particles were stable for around 10 h at 37 °C and pH 7.4. DNA polyplexes based on this polymer are stable in serum and show a high in vitro transfection activity with minimal cytotoxicity [11].

Trimethylated chitosan (TMC) has been studied for DNA and siRNA delivery [12]. The degradation of TMC is due to hydrolysis of the glycosidic bond (indicated with an arrow in Fig. 1) [13].

Our group recently reported on the synthesis of a well defined and enzymatically degradable TMC (O-methyl-free N,N,N-trimethylated chitosan) (Fig. 1) [14].

Polyplexes can be taken up by cells through endocytosis [15,16]. Particles entering the cell through this route become entrapped in

* Corresponding author. Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands. Tel.: +31 30 2537388; fax: +31 30 2517839.
E-mail address: G.Storm@uu.nl (G. Storm).

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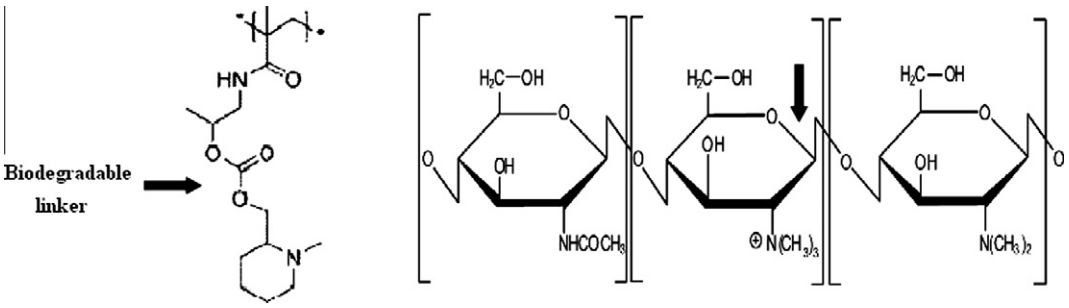

Fig. 1. Structure of pHPMA-MPPM (left, poly(hydroxy propyl methacrylamide-1-methyl-2-piperidine methanol) and TMC (right, O-methyl-free N,N,N-trimethylated chitosan). In TMC, the various substituents are randomly distributed over the polymer chain (acetyl groups (see block 1), quaternized amines (see block 2)).

endosomes and eventually end up in lysosomes where active degradation processes under the action of special enzymes take place [17]. Thus, one of the crucial steps in successful nucleic acid delivery with cationic polymers is the escape of the polyplexes from endosomes [18]. Several approaches have been used to promote the endosomal escape of endocytosed polyplexes. One of these approaches is Photochemical Internalization (PCI), a technique based on the use of photosensitizers that localize in endosomal membranes upon addition to cells and photochemically destabilize endosomal membranes after illumination, with subsequent release of endocytosed material into the cytosol [19]. In addition, several endosomal membrane-disrupting peptides such as diINF-7, a peptide based on the fusion domain of the influenza virus, have been used to facilitate the endosomal escape of polyplexes. At endosomal pH, at which such peptides become fusogenic, the C-terminal side of the V-shaped peptide undergoes a conformational change, resulting in the formation of a helical structure that allows insertion of the peptide into the lipid bilayer, leading to endosomal membrane destabilization [20].

In this study, pHPMA-MPPM and TMC were investigated for siRNA delivery. We compared these polymers with the non-degradable polymers poly(2-dimethylamino)ethyl methacrylate) (pDMAEMA) [21] and polyethylenimine (PEI) and the routinely used commercially available lipidic transfection agents Lipofectamine. In addition, the application of PCI and the use of the membrane-disrupting peptide diINF-7 on the silencing activity of the polyplexes were investigated.

2. Materials and methods

2.1. Materials

The Fluc double-stranded siRNA that specifically targets firefly luciferase (used against mRNA from pGL3; structure below), anti-EGFP siRNA as non-specific siRNA and Fluc TYE563-labeled siRNA were obtained from Integrated DNA Technologies BVBA, Leuven, Belgium.

Sense strand: 5′-pGGUUCCUGGAACAAUUGCUUUUAca.
Antisense strand: 3′-GACCAAGGACCUUGUUAACGAAAAUGU.

Branched polyethylenimine (Mw 50–100 kDa) was obtained from Polysciences, Inc. (Warrington, Pennsylvania USA). PHPMA-MPPM (240 kDa) [11], TMC 33% (60 kDa) [14] and Poly(2-(dimethylamino) ethylmethacrylate) (pDMAEMA) [21] were synthesized and purified as described previously. Sodium heparin and gelatin solution were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) and Lipofectamine 2000 was obtained from Invitrogen (Breda, The Netherlands). Luciferase assay reagent and reporter lysis buffer were obtained from Promega (Leiden, The Netherlands). Formaldehyde was obtained from Fluka (Zwijndrecht, The Netherlands), and FluorSave for mounting the cells onto glass cover slides was obtained from Calbiochem (San Diego, CA, USA). INF-7 was synthesized as previously described [20]. Its fusogenic activity was assessed using a liposome leakage assay [22]. The photosensitizer (PS) TPPS2a, meso-tetraphenylporphyrin, was obtained from PCI Biotech AS (Oslo, Norway) [23]. LumiSource®, a bank of four light tubes emitting light with a wavelength range of 375–450 nm and 13 mW/cm^2 irradiation intensity, was provided by PCI Biotech AS, Oslo, Norway.

2.2. Physicochemical characterization of polyplexes

Polyplexes with different polymer to siRNA ratios (expressed as *N*/*P* ratios, where N is the moles of cationic nitrogens in the polymer and P is moles of phosphate in siRNA) between 0.5 and 20 were prepared by adding a Fluc siRNA solution (175 μl, 20 μg/mL) in HEPES (5 mM, pH 7.4) to polymer solutions (700 μl, various concentrations) in the same buffer. The resulting solutions were vortexed for 10 s. After 30 min incubation at room temperature, *Z*-average diameters were measured with dynamic light scattering at 25 °C with a Malvern 4700 system using an argon-ion laser (488 nm) operating at 10.4 mW (Uniphase) and PCS (photon correlation spectrometry) software for Windows version 1.34 (Malvern, UK). Viscosity and refractive index of water at 25 °C were used. The system was calibrated with an aqueous dispersion of polystyrene particles with a diameter of 100 nm. Particle size distribution is described using the polydispersity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of the polyplexes prepared in HEPES (5 mM, pH 7.4) was determined at 25 °C in a DTS5001 cell with a Zetasizer 2000 unit (Malvern). The instrument was calibrated with a polystyrene dispersion with known zeta potential. In some experiments, after 30 min incubation of polyplexes at room temperature, 50 μl aqueous solutions of INF-7 peptide (different concentrations) in HEPES (5 mM, pH 8) was added to the polyplex dispersion (final peptide concentration ranged from 10 to 180 μg/mL). After vortexing for 5 s, the dispersions were incubated at room temperature for 15 min and characterized for size and charge. The colloidal stability of the polyplexes in HEPES (5 mM, pH 7.4) was studied by measuring their *Z*-average diameters during storage at room temperature.

2.3. Agarose gel electrophoresis

The complexation of siRNA with the various transfectants under study and the heparin-induced destabilization of the polyplexes were investigated using agarose gel electrophoresis. The agarose gels (NuSieve® GTG® Agarose, Lonza, Rockland, ME, USA) were made in a concentration of 4% (w/v) in Tris–Acetate-EDTA (TAE) running buffer and contained 0.5 μg/mL ethidium bromide. Polyplexes (*N*/*P* ratios varying from 0.5 to 20) of different polymers with Fluc siRNA were prepared as described above. Naked siRNA

was also used. Polyplex dispersions (*N*/*P* 8) with or without heparin (0.4–2.7 USP/μg siRNA; molar ratio of amine groups of the polymer and sulfate groups of heparin (*N*/*S*) varied from 0.4 to 3) were incubated for 5 min at room temperature. Polyplexes (15 μl, corresponding with 15 pmol siRNA) were applied in the starting slot of the gel, and electrophoresis was performed at 60 V for 50 min. The siRNA bands stained with ethidium bromide were detected on a UV transilluminator (ImaGo compact imaging system (B&L Systems), The Netherlands).

2.4. Gene silencing and cytotoxicity experiments

The human lung cancer cell line H1299 that expresses firefly luciferase was used to study the gene silencing activity of the different polyplex formulations. The cell line was maintained in RPMI 1640 medium with HEPES and L-glutamine (PAA laboratories GmbH, Pasching, Austria, catalog No. E15-842) completed with fetal bovine serum (FBS) (final concentration 10% v/v) and cultured at 37 °C at 5% CO_2 humidified atmosphere. The cells (8×10^3 cells/well) were seeded into 96-well plates and cultured overnight. Different concentrations of photosensitizer (ranging from 0.05 to 1.5 μg/mL) and different illumination times were applied (15–90 s). To achieve the strongest silencing with minimal cytotoxicity (⩽10%), we selected a dose of photosensitizer of 0.5 μg/mL and illumination time of 75 s. One day after seeding, the cells were incubated at 37 °C with complete culture medium containing photosensitizer for 18 h. Next, the medium was removed, and fresh medium was added to the wells, and the anti-Luciferase siRNA polyplexes (10 pmol siRNA/well) were added to the cells and incubated at 37 °C for 4 h. Then, the medium was removed, fresh medium was added, and the cells were exposed to the light source for 75 s. Subsequently, the cells were incubated at 37 °C for 2 days, after which the luciferase protein expression was analyzed using Luciferase reporter gene assay (Promega). The cytotoxicity of the polyplexes was measured using the XTT colorimetric viability assay as previously described [24]. When the PCI treatment was not applied, the day after seeding, the cells were incubated with polyplexes with or without INF-7 peptide (final concentration of 1.5–9.5 μg/mL in growth medium) for 4 h, followed by refreshing the medium and 2 days incubation at 37 °C. Polyplexes of specific and non-specific siRNA with Lipofectamine 2000 were prepared by gently mixing 50 μl siRNA (20 μg/mL) in HEPES (5 mM, pH 7.4) with 3.7 μl Lipofectamine 2000 in 50 μl HEPES (5 mM, pH 7.4) followed by 30 min incubation at room temperature.

2.5. Determination of luciferase activity

Luciferase activity was measured after removal of the growth medium and lysis of the cells by the addition of 100 μl reporter gene lysis buffer. After a freeze/thaw cycle at −80 °C/room temperature, 50 μl of luciferase assay reagent was added to 50 μl of the cell lysate, and relative light units (RLU) were measured for 10 s at room temperature using a FLUOstar OPTIMA microplate-based multi-detection reader with a microinjector.

2.6. Multi-photon laser scanning microscopy

The cellular uptake of polyplexes was investigated with multiphoton laser scanning microscopy (MPLSM). In short, 8×10^3 cells were seeded in the wells of 12-well plates, pre-coated with 100 μl gelatin. The cells were cultured for 24 h at 37° C and subsequently incubated with Fluc TYE563-labeled siRNA lipo/polyplexes (with or without photosensitizer (PS)). Then, the cells were fixed with 4% solution of formaldehyde in PBS for 30 min at room temperature. After fixation, the cells were washed twice with PBS and incubated with DAPI nucleic acid stain (300 nM) in PBS for 3 min. Subsequently, the cells were washed twice with PBS and mounted onto glass cover slides, using FluorSave. The cells were analyzed using MPLSM (Bio-Rad, Hemel Hempstead, United Kingdom). Excitation of DAPI was achieved by multiphoton excitation at 780 nm using mode-locked Titanium: Sapphire laser (Tsunami; Spectra Physics, San Jose, CA) pumped by a 10-W solid state laser (Millennia Xs; Spectra Physics), whereas TYE563 was excited by confocal laser light (550 nm). Samples were viewed with a TE200 inverted microscope using a 60/1.4 oil objective (Nikon, Tokyo, Japan). Images were analyzed using LaserSharp 2000 software (Bio-Rad).

3. Results and discussion

In this study, pHPMA-MPPM and TMC were investigated for their siRNA delivery properties and compared with those of well-known non-degradable polymeric transfectants (pDMAEMA) [21], branched PEI [25,26] and the cationic lipid Lipofectamine [27].

3.1. Physicochemical characterization of polyplexes

The average diameter of the polyplexes prepared at different *N*/*P* ratios were measured by dynamic light scattering (Fig. 2). All polymers were capable of forming complexes with siRNA. In case of the PEI- and pHPMA-MPPM-based polyplexes, small particles with an average size around 100–150 nm were formed over the whole *N*/*P* range of 0.5–20. The average size of pDMAEMA-based and TMC-based polyplexes decreased with increasing *N*/*P* ratio, with small-sized nanoparticles of 100–150 nm being formed at *N*/*P* ratio ⩾8. This can probably be ascribed to an increased polymer amount in the formulations which leads to tighter complexes. Furthermore, a polydispersity index (PDI) of ⩽0.3 was observed for all polyplex formulations made at *N*/*P* ratio ⩾8, which indicates a

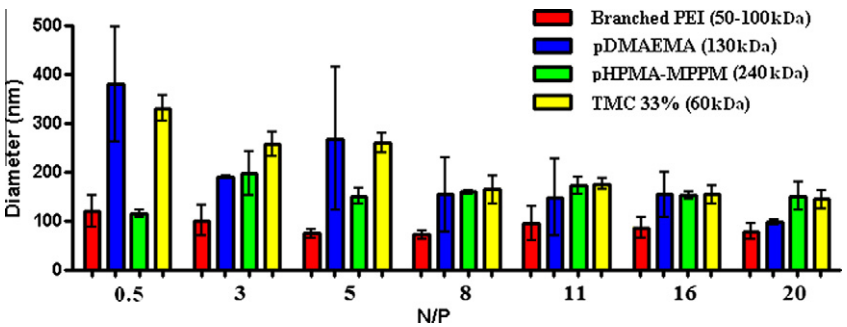

Fig. 2. Average diameter of polyplexes prepared at different *N*/*P* ratios (mean ± standard deviation (*n* = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rather narrow size distribution. At lower N/P ratios, the pDMAEMA and TMC-based polyplexes were significantly larger than pHPMA-MPPM and PEI-based polyplexes. In case of TMC, this is most likely due to the lower charge density of TMC when compared to pHPMA-MPPM and PEI.

At N/P of 0.5, the different polyplexes had a negative zeta potential (about −25 mV), which can be explained by the excess of siRNA present in the formulations. At $N/P \geqslant 3$, positively charged particles (zeta-potential around 20 mV, Supporting information S1) were formed as a result of the increased polymer amount in the formulations.

3.2. Agarose gel electrophoresis

Binding of cationic polymers to siRNA molecules and subsequent complex formation are essential for protection of siRNA against degradation by endonucleases. On the other hand, polyplex destabilization also needs to occur as siRNA should be released in the cytosol in order to achieve gene silencing activity. Heparin is commonly used as a model anionic polyelectrolyte to monitor the sensitivity of polyplexes for dissociation and release of siRNA [28,29].

Fig. 3 shows that siRNA remains in the starting slots of the gel for siRNA/polymer formulations prepared at $N/P \geqslant 3$, indicating, in agreement with DLS analysis (Fig. 2), that polyplexes were formed. In line with previous studies [30,31], no siRNA was detected in case of the PEI formulations (Fig. 3d) pointing to the formation of strong complexes. After incubation of the polyplexes with heparin, dissociation of polyplexes and subsequent release of the siRNA was observed, except for the PEI/siRNA polyplexes, again indicating that PEI and siRNA form strong complexes. Fig. 3a shows that polyplexes based on pHPMA-MPPM are dissociated at the lowest heparin concentration investigated ($N/S = 3$) pointing to a low binding strength between this polymer and siRNA. In case of the TMC-based polyplexes, release of the siRNA was observed at $N/S \leqslant 1$ (Fig. 3b) which indicates a higher binding strength of TMC to siRNA than pHPMA-MPPM. Fig. 3c shows that siRNA is released from pDMAEMA polyplexes at $N/S \leqslant 1.8$ which indicates an intermediate binding strength between this polymer and siRNA.

3.3. Silencing activity and cell viability

Human lung cancer cells (H1299) expressing luciferase were used for gene silencing studies. Polyplexes prepared at N/P ratios of 8 and 16 were selected for silencing studies, because these particles showed binding to the siRNA (Fig. 3), had a positive mean zeta potential (Supporting information S1), and small average size (Fig. 2). Fig. 4a shows that in the absence of serum, incubation of cells with polyplexes based on pDMAEMA resulted in up to 50% silencing activity, whereas incubation with pHPMA-MPPM and TMC polyplexes made at N/P ratios of 8 and 16 showed 30–40% gene silencing. The activity of these polyplexes is comparable with the lipofectamine formulation (60% silencing efficiency). Incubation of cells with polyplexes based on branched PEI led to 25–30% silencing of luciferase. In all cases, the effect of the N/P ratio (8 vs 16) was small. The relatively low silencing activity of

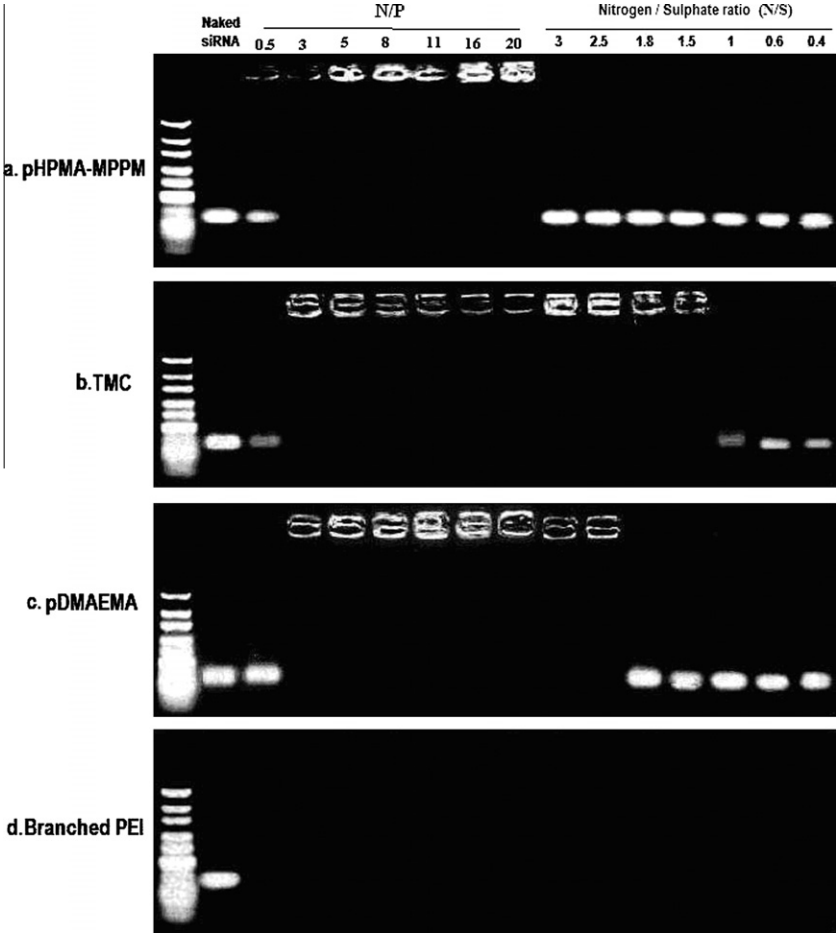

Fig. 3. Agarose gel electrophoresis. Polyplexes made in 5 mM HEPES buffer (pH: 7.4) at N/P ratios 0.5–20 were applied on a 4% agarose gel. To study polyplex destabilization and siRNA release, polyplexes made at an N/P ratio of 8 were incubated for 5 min with heparin solution (different concentrations) and subsequently applied on the gel.

branched PEI/siRNA polyplexes can likely be explained by the strong binding of PEI to the siRNA which inhibits the liberation of the siRNA from the complexes (Fig. 3d). Fig. 4b shows low cytotoxicity of the lipofectamine and polymer formulations made at an *N*/*P* ratio of 8 (<15%) (except PEI formulation, ~25%). When compared to a *N*/*P* of 8, the cytotoxicity of polyplexes prepared at an *N*/*P* ratio of 16 was about two fold higher (in case of PEI less than 2-fold) which can be ascribed to the higher concentration of the cationic polymers in the formulations. In this study, all the gene silencing results were corrected for cytotoxicity.

Fig. 4c shows that, importantly, the gene silencing activity of pHPMA-MPPM, TMC and pDMAEMA polyplexes was not affected by the presence of serum proteins. Moreover, in the presence of serum, the cytotoxicity of the polyplexes was slightly lower (Fig. 4d) than that of the complexes incubated with the cells in medium without serum (Fig. 4b). As suggested in literature, serum proteins might mask the cytotoxicity of cationic polyplexes and polymers [32,33].

3.4. Enhancement of endosomal escape

PCI is a technique based on the use of photosensitizers that photochemically destabilize endosomal membranes after illumination [34,35]. It appears that by application of PCI, the gene silencing activity of the pHPMA-MPPM and TMC polyplexes increased from 30–40% (Fig. 4a) to ~70–80% (Fig. 5a). This means that the endosomal escape properties of these polymers are limited, as previously reported for pHPMA-MPPM/DNA complexes [11]. By application of PCI, the silencing efficiency of the pDMAEMA polyplexes only slightly increased which confirms previous findings that pDMAEMA polyplexes have intrinsic endosomal escape properties [36]. In a recent study by Boe et al., it was reported that gene silencing activity of PEI/siRNA complexes was enhanced after application of PCI [37]. In our study, the gene silencing activity of polyplexes based on PEI did not benefit from PCI treatment. In comparison with the study of Boe et al., we used PEI with a higher molecular weight (50–100 kDa) which likely forms stronger complexes, with less release of the siRNA as confirmed by the heparin competition assay in Fig. 3. Also, the cell line which we used was different than the one used in the study of Boe et al. For Lipofectamine-based complexes, silencing activity increased up to 70% after application of PCI (Fig. 5a). Importantly, the application of PCI was not associated with an increase in cytotoxicity (compare Figs. 4b and 5b).

The effect of PCI on gene silencing activity of the siRNA complexes was also studied in the presence of serum in the medium. In the presence of serum, also an increased gene silencing activity was observed after application of PCI for the TMC, pHPMA-MPPM and pDMAEMA-based polyplexes (Supporting information S2a, compare with Fig. 4c). In line with the results of Fig. 5a, PCI has no effect on the activity of the PEI-based polyplexes. The cytotoxicity of PEI-based and pDMAEMA-based polyplexes after application of PCI was lower than in the absence of serum (Supporting information S2b).

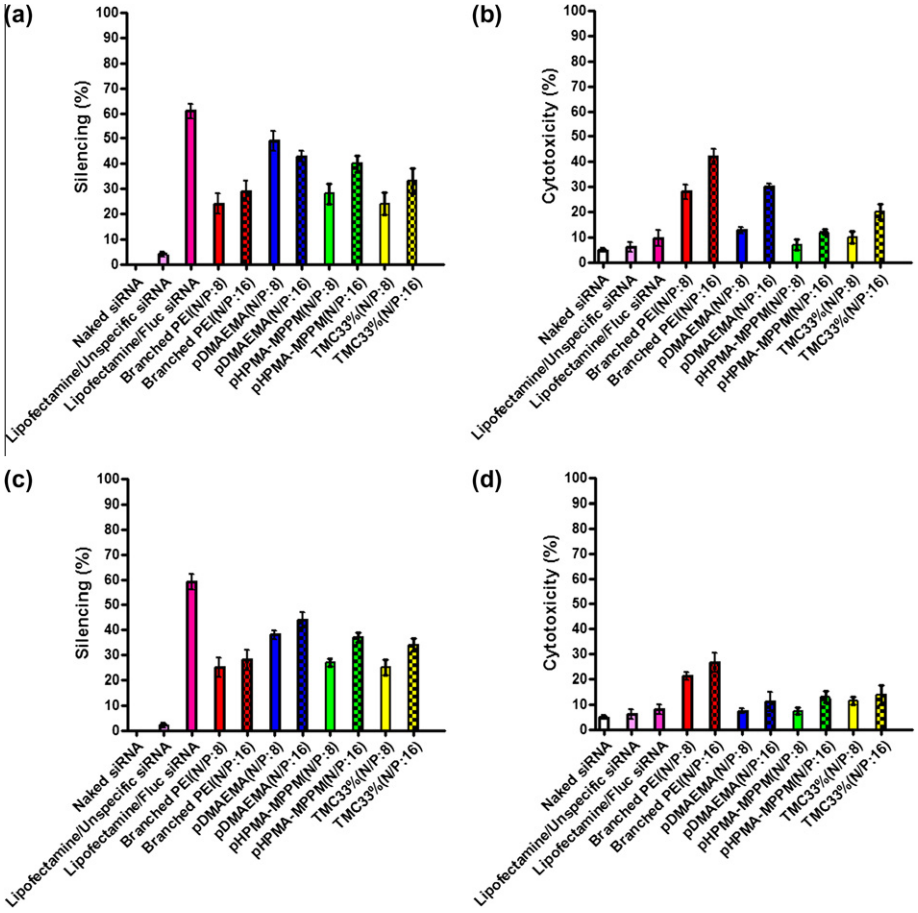

Fig. 4. (a) Luciferase gene silencing after incubation of H1299 cells with siRNA complexes in serum-free medium. (b) Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in serum-free medium. (c) Luciferase gene silencing of siRNA complexes after incubation with H1299 cells in the presence of 10% FBS. (d) Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes in the presence of 10% FBS (mean ± standard deviation (n = 3)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

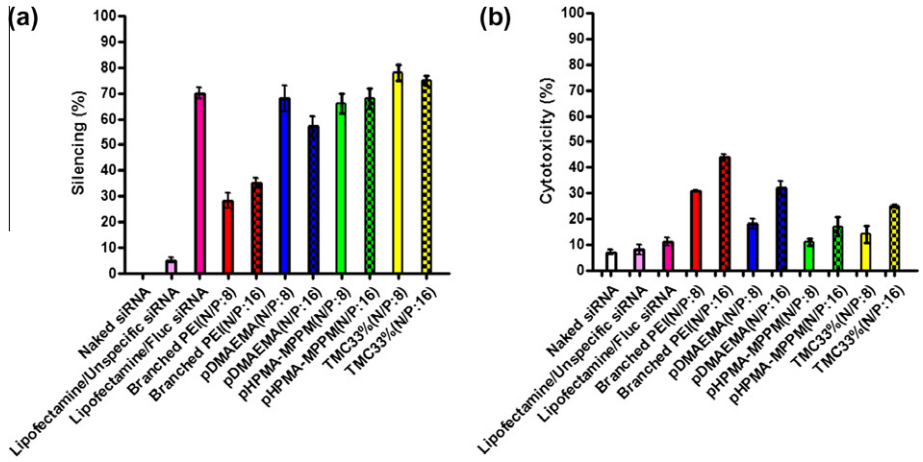

Fig. 5. (a) Effect of PCI on luciferase gene silencing activity of siRNA complexes after incubation with H1299 cells in serum-free medium. (b) Cell viability as measured by XTT assay after application of PCI for cells incubated with siRNA complexes in serum-free medium (mean ± standard deviation (n = 3)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Besides by PCI, endosomes can be destabilized by endosome disruptive peptides. Therefore, we studied the effect of the endosome disruptive diINF-7 peptide on gene silencing efficiency of pHPMA-MPPM polyplexes. It was shown that this peptide is able to destabilize liposomes at pH 5 (Supporting information S3) [18,20,38,39]. DLS measurements showed that incubation of the polyplexes with the diINF-7 peptide did not affect their size distribution (data not shown). On the other hand, a gradual decrease in zeta potential of the polyplexes with increasing concentrations of diINF-7 was observed. At high concentrations of the peptide, the polyplexes became even negatively charged (Supporting information S4). At pH 7, diINF-7 is negatively charged and therefore binds via electrostatic interactions to the positively charged surface of the polyplexes. At high concentrations of the peptide, the surface is likely fully covered with the peptide by which the zeta potential of the polyplexes is reversed.

The gene silencing activity of diINF-7-coated pHPMA-MPPM polyplexes is shown in Fig. 6a. The silencing activity of the Lipofectamine formulation slightly increased with increasing diINF-7 concentration (from about 55% to 70%). Incubation of cells with pHPMA-MPPM formulations containing 25–100 μg/mL diINF-7 peptide resulted in a gradual increase of the silencing activity from 25% to 50%, again indicating that the pHPMA-MPPM polyplexes have limited endosomal escape tendency. At the highest concentration of peptide (145 μg/mL) used, a drop in silencing activity to about 30% was observed, which may be explained by a too strong reduction in the zeta potential with less cellular interaction and uptake as a consequence [40,41] (Fig. 6a). The XTT cell viability assay shows low cytotoxicity of the diINF-7-coated siRNA complexes (Fig. 6b).

3.5. Multi-photon laser scanning microscopy

Multi-photon laser scanning microscopy (MPLSM) was used to study the cellular uptake and internalization of siRNA complexes using fluorescently labeled siRNA. Naked siRNA was not taken up by cells (not shown), in agreement with previous studies. Fig. 7 shows that red spots originating from the labeled siRNA (images d and h) were intracellularly observed after incubation of the cells with the various siRNA formulations investigated in this study, demonstrating that the complexes were internalized. The fluorescence patterns did not change after application of PCI, even for polyplexes for which a PCI-mediated increase in gene silencing efficiency was noted (Fig. 5a and Supporting information S2a).

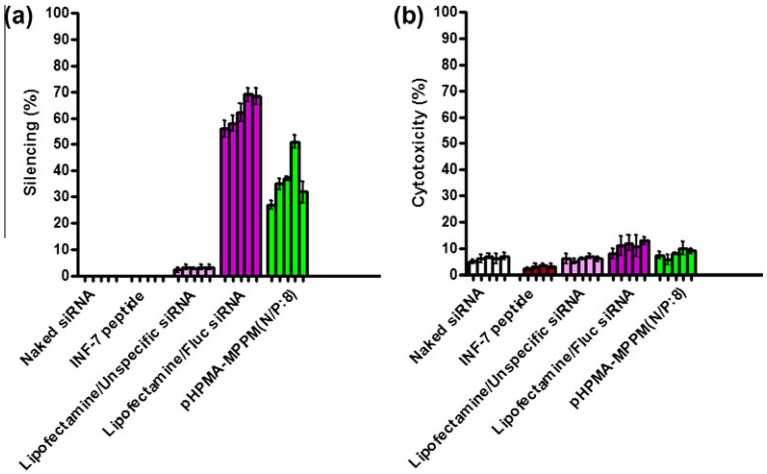

Fig. 6. (a) Luciferase gene silencing after incubation of H1299 cells with siRNA complexes, with or without diINF-7 peptide in the medium containing 10% FBS. (b) Cell viability as measured by XTT assay after incubation of H1299 cells with siRNA complexes, with or without diINF-7 peptide, in the medium containing 10% FBS. In all formulations, the first bar shows the polyplexes without diINF-7, and second, third, fourth and fifth bars show the polyplexes containing 25, 50, 100 and 145 μg/ml diINF-7 (mean ± standard deviation (n = 3)). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

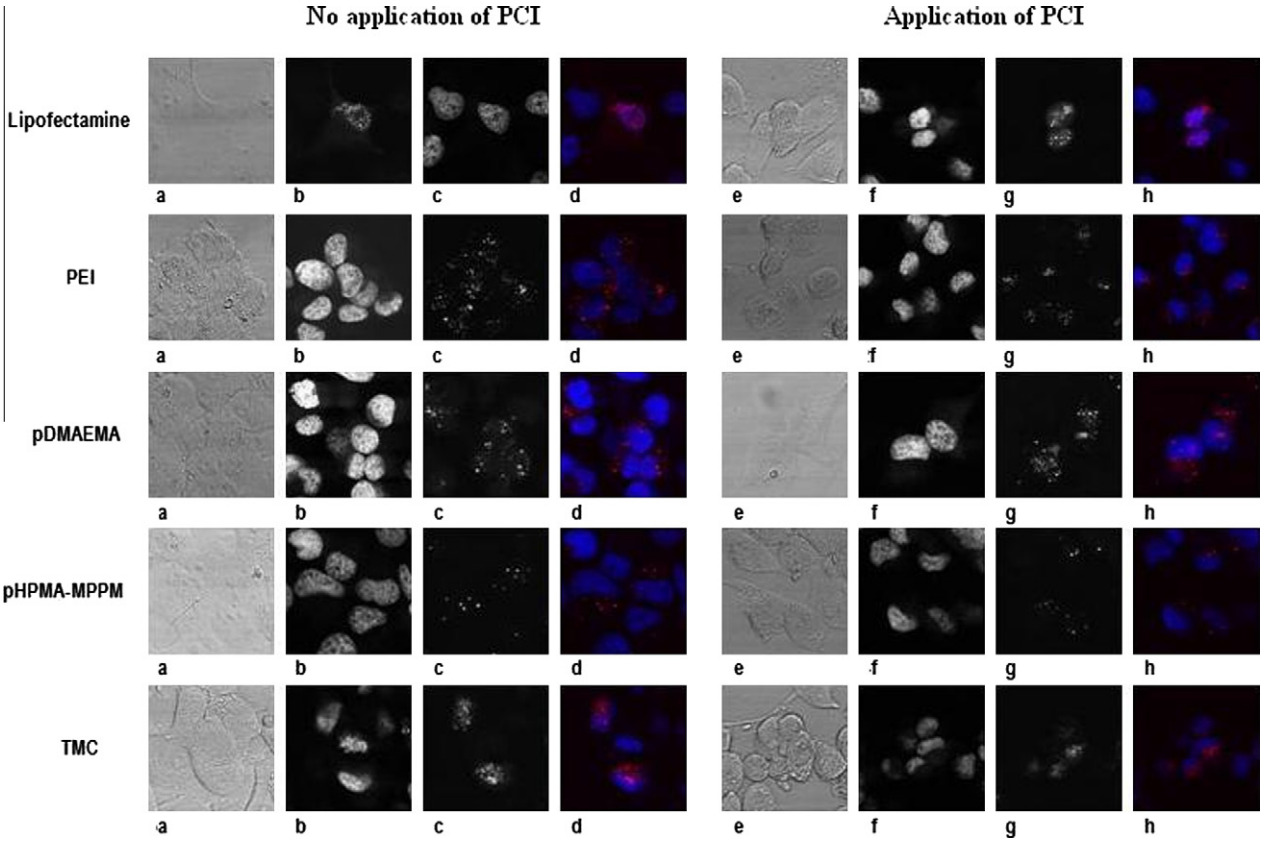

Fig. 7. Multi-photon laser scanning microscopy images of H1299 cells incubated with complexes prepared with TYE 563-labeled siRNA (red). Images **a** and **e**: Pattern of cells with light microscopy, Images **b** and **f**: Nuclei of cells stained with Dapi, Images **c** and **g**: Complexes with TYE 563-labeled siRNA, Images **d** and **h**: Red, Complexes with TYE 563-labeled siRNA; Blue, DAPI stained nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

These results indicate that the PCI effect is below the detection limit of the MPLSM technique. The PCI effect is difficult to visualize as the punctuate fluorescence signal from endocytic vesicles is lost upon PCI when the siRNA appears through the cytosol, as it was shown previously by Oliveira et al. [42]. The images of cells treated with Lipofectamine/siRNA complexes do visualize release of siRNA into the both cytosol and nucleus, in agreement with previous observations [30,43].

4. Conclusion

This study introduces biodegradable pHPMA-MPPM and TMC as promising vectors for siRNA delivery. When compared to the non-degradable PEI- and pDMAEMA-bases polyplexes, their silencing activity was similar but their cytotoxicity profile more favorable. By promoting the endosomal escape of these polyplexes using either PCI or an endosome disruptive peptide, enhancement of their silencing activity was observed. Importantly, the polyplexes preserve their gene silencing activity in the presence of serum proteins, while their cytotoxicity remained low. These features make these polymers attractive candidates for further development and warrant application in animal studies.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.11.016.

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