International Journal of Pharmaceutics 525 (2017) 388–396

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Small nanosized poly(vinyl benzyl trimethylammonium chloride) based polyplexes for siRNA delivery

Bo Lou[a], Nataliia Beztsinna[a], Grigoris Mountrichas[b], Joep B. van den Dikkenberg[a], Stergios Pispas[b], Wim E. Hennink[a,*]

[a] Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Universiteitsweg 99, 3584 CG, Utrecht, The Netherlands
[b] Theoretical and Physical Chemistry Institute, National Hellenic Research Foundation, 48 Vass. Constantinou Ave., 11635 Athens, Greece

ARTICLE INFO

Article history:
Received 13 January 2017
Received in revised form 17 March 2017
Accepted 18 March 2017
Available online 20 March 2017

Keywords:
Quaternized polymer
siRNA
Polyplexes
Polymeric gene delivery
Gene therapy

ABSTRACT

The success of siRNA gene therapy requires the availability of safe and efficient delivery systems. In the present study, we investigated poly(vinyl benzyl trimethylammonium chloride) (PVTC) and its block copolymer with poly(oligo(ethyleneglycol) methacrylate) (POEGMA) as delivery vector for siRNA. Small polyplexes ranging from 8 to 25 nm in diameter were formed in aqueous solution by spontaneous self-assembly of both the homopolymer and block copolymer with siRNA and the formed particles were stable at physiological ionic strength. It was shown that when human ovarian adenocarcinoma cells were transfected, siRNA polyplexes based on PVTC (40 kDa) and PVTC-POEGMA-4 (PP4, 34 kDa) efficiently induced luciferase gene silencing to the same extent as the formulation based on a commercial lipid (Lipofectamine®) (~80%), and showed higher gene silencing than the linear polyethylenimine formulation linear polyethylenimine (~35%). Importantly, the POEGMA block polymers displayed a significantly lower cytotoxicity as compared to L-pEI. siRNA polyplexes based on the block polymers displayed high cellular uptake resulting in ~50% silencing of luciferase expression also in the presence of serum. These results demonstrate that PVTC-based polymers are promising siRNA delivery vectors.

1. Introduction

In the past 15 years, RNA interference opened exciting therapeutic possibilities and large investments have been made for its use to treat various diseases, including viral infections and cancer (Kole et al., 2012; Whitehead et al., 2009). siRNA, a short double stranded oligonucleotide with 21–23 base pairs, is a highly water-soluble polyanion (Mw around 13 kDa), which is rapidly degraded by nucleases in the extracellular environment (Carthew and Sontheimer, 2009). Moreover, siRNAs are unable to spontaneously pass cellular membranes as both the oligonucleotide and cellular membrane are negatively charged. Since RNA interference has to occur in the cytoplasm, it is crucial for siRNA to be presented in the cytosol for exerting its pharmacological activity. Therefore, safe and highly efficient siRNA delivery systems, which protect these nucleic acid based drugs from enzymatic degradation and assist in overcoming the delivery barriers are required.

Polymer-based non-viral vectors, especially cationic polymers, have been widely studied for siRNA delivery both *in vitro* and *in vivo*. These polymeric delivery systems are known to have several advantages such as safety, easy large-scale production, and absence of immune side effects as compared to viral vectors. Cationic polymers form nano-sized complexes with negatively charged siRNAs and these complexes can be, depending on their biophysical properties such as size and charge, internalized by living cells. A great variety of both natural and (semi) synthetic cationic polymers has been investigated for nucleic acid delivery, including poly(L-Lysine) (Oe et al., 2014; Zauner et al., 1998), poly (ethylene imine) (Boussif et al., 1995; Liu et al., 2016), PDMAEMA (Cherng et al., 1996; Van de Wetering et al., 1999), poly(amido amine)s (Lin et al., 2008), cationic cyclodextrins (Davis et al., 2010) and chitosan (Sinha and Kumria, 2001). The gene binding capacities and the transfection abilities of these and other polymers have been summarized in a number of review papers (Chen et al., 2016; Lachelt and Wagner, 2015; Wong et al., 2007).

* Corresponding author.
E-mail address: W.E.Hennink@uu.nl (W.E. Hennink).

http://dx.doi.org/10.1016/j.ijpharm.2017.03.036
0378-5173/

However, one major obstacle for the translation of non-viral gene delivery systems into clinical application is their colloidal stability. Cationic polyplexes tend to aggregate/dissociate in the presence of proteins and other polyanions in blood and other biological fluids, which leads to premature nucleic acid release (Arigita et al., 1999; Dash et al., 1999). A commonly used approach to tackle the poor colloidal stability of the first generation polyplexes is their surface modification with a neutral and hydrophilic polymer [e.g., poly (ethylene glycol) (PEG)] that shields their charge and reduces nonspecific interactions (Kircheis et al., 2001; Ogris et al., 1999; Suk et al., 2016; Zoetebier et al., 2016). However, several studies have shown that PEGylated polyplexes still exhibited undesirable release of siRNA payloads and displayed poor pharmacokinetics in animal models (Christie et al., 2012; de Wolf et al., 2007; Kim et al., 2014; Malek et al., 2009). To increase the colloidal stability of PEGylated polyplexes, several sophisticated methods have been developed including reversible cross-linking for spatio-temporal release (Lin et al., 2006; Novo et al., 2015) and hydrophobic interactions (Kim et al., 2012; Nelson et al., 2013; Oe et al., 2014). Apart from this, several studies have indicated that the simple conversion of primary into quaternary amines or the use of polymers already containing quaternary amine groups could enhance the polymer-siRNA binding strength and lead to the particles that are more colloidally stable in physiological fluids. (Amar-Lewis et al., 2014; Arigita et al., 1999; Delisavva et al., 2013; Engelberth et al., 2015; Patil et al., 2008; Tamura et al., 2010).

Recently, a novel polymer, poly(vinyl benzyl trimethyl-ammonium chloride) (PVTC) containing permanently quaternized amines and a hydrophobic benzyl groups as backbone, was synthesized by RAFT polymerization (Haladjova et al., 2016). This study showed that this quaternized polymer formed complexes with plasmid DNA with a size ranging of 80–300 nm and with a good particle stability even in the presence of 300 mM sodium chloride. It is expected that these PVTC-based polymers could also form stable particles with siRNA and ensure its successful intracellular delivery. In this study, we characterized the physico-chemical properties of polyplexes formed with the homopolymer PVTC and block copolymers POEGMA-PVTC and evaluated the ability of these polyplexes to deliver siRNA into the cells.

2. Materials and methods

2.1. Materials

The double-stranded anti-luc siRNA-1(D-002050-01) which specifically targets firefly luciferase (used against mRNA from pGL3; sense strand: 5′- CUU ACG CUG AGU ACU UCGA - 3′), siGLO Red(DY-547) Transfection Indicator (D-001630-02) and Non-target siRNA-1(D-001810-01) were obtained from Dharmacon Bioscience (Lafayette, USA).

The homopolymer poly(vinyl benzyl trimethyl-ammonium chloride) (referred as PVTC) and two different poly[oligo(ethyl-eneglycol) methacrylate]-b-poly(vinyl benzyl trimethylammonium chloride) block copolymers (referred as PP2 and PP4) were synthesized by RAFT polymerization as described previously (Haladjova et al., 2016). Their molecular characteristics are shown in Table 1. Linear polyethylenimine (L-pEI, Mw 25,000) was obtained from Polysciences and Lipofectamine 2000 was from Thermo Fisher Scientific (Etten-Leur, The Netherlands).

Agarose multi-purpose was purchased from Roche Molecular Biochemicals (Mannheim, Germany). 6 × DNA Loading Dye was purchased from Fermentas (St. Leon-Roth, Germany). SYBR Safe DNAgel stain, Opti-MEM, DMEM medium and dialyzed fetal bovine serum (FBS) were purchased from Life Technologies (Breda, The Netherlands). Skov3-luc (human ovarian carcinoma cell line stably expressing firefly luciferase) cells were obtained from CellBioLabs (San Diego, USA). Luciferase assay kit was purchased from Promega (Leiden, The Netherlands). All other chemicals, reagents and media were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2. Preparation and characterization of polymer/siRNA polyplexes

Polymer/siRNA polyplexes of nitrogen/phosphate (N/P) ratios from 0.5 to 16 were prepared by adding 400 μL of polymer solution (varying concentrations in 10 mM HEPES buffer, pH 7.4) to 200 μL of siRNA (40 μg/mL in 10 mM HEPES buffer, pH 7.4), followed by vortexing for 5 s and incubating at room temperature for 30 min. Particle sizes were measured with dynamic light scattering (DLS) using an ALV CGS-3 system (Malvern Instruments, Malvern, U.K.) equipped with a JDS Uniphase 22 mW He−Ne laser operating at 632.8 nm, an optical fiber-based detector, and a digital LV/LSE-5003 correlator with temperature controller set at 25 °C. The zeta-potential of the polyplexes was measured at 25 °C using a Malvern Zetasizer Nano-Z (Malvern Instruments, Malvern, U.K.).

2.3. Gel retardation assay

Polyplexes prepared at different N/P ratios were made by adding 5 μL of polymer solution (varying concentration 14–2400 μg/mL in 10 mM HEPES, pH 7.4) to 5 μL of siRNA solution (40 μg/mL in 10 mM HEPES, pH 7.4), followed by vortexing for 5 s and the dispersions were incubated for 30 min at room temperature. For release studies with heparin, the sample prepared at N/P of 8 was mixed with of 5 μL heparin sodium salt (varying concentration 75–900 μg/mL). Next, 1 μL of sodium chloride (1.54 M) was added to get a final salt concentration of 150 mM, and the samples were incubated for 10 min at room temperature (Nuhn, 2014). After the addition of 3 μL of 6× loading buffer (Fermentas), the mixture was loaded into a 2% agarose gel containing GelGreen (Biotium) in a tris-acetate-EDTA (TAE) buffer and the gel was developed at 100 V for 30 min. Next, the gel was analyzed by a Gel Doc XR+ system (BioRad Laboratories Inc., Hercules, CA) with Image Labsoftware.

2.4. Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) was carried out using a FEI Tecnai T10 microscope from FEI company (Eindhoven, Netherland). Twenty microliters of polyplexes dispersion at 20 μg/mL siRNA in 10 mM HEPES, pH 7.4, was placed on a carbon-coated copper grid. The samples were stained with 2% uranyl acetate. Scale bars were added with a help of ImageJ software.

Table 1
Characteristics of the PVTC-based homo- and block copolymers used in this study.

code	name	M_n (g mol^{-1})	PVTC M_n (g mol^{-1})	POEGMA M_n (g mol^{-1})	PVTC% (w/w)	M_w/M_n
PVTC	PVTC-40k	39,600	39,600		100	1.28
PP2	PVTC-POEGMA-2	22,400	4200	18,200	19	1.21
PP4	PVTC-POEGMA-4	33,600	15,400	18,200	46	1.24

2.5. Cell culture

Skov3-luc cells which stably express firefly luciferase, were cultured in DMEM (4.5 g/L glucose) supplemented with 10% FBS (Sigma-Aldrich). Cells were maintained at 37 °C in a 5% CO_2 and humidified air atmosphere.

2.6. In vitro *luciferase gene silencing efficiency assay*

Skov3-luc cells were plated in 96-well plates (7×10^3 cells/well) cultured in full medium for at least 24 h until 60–70% cell confluence was reached. The cells were then washed with PBS buffer and subsequently incubated in medium with or without 10% serum. Next, the siRNA polyplexes (anti-luciferase or negative control) of varying N/P ratios were prepared as described in section 2.2 (10 mM HEPES buffer, pH 7.4) and added to the cells (siRNA concentration was 100 or 200 nM) and incubated for 4 h at 37 °C in a 5% CO_2-containing atmosphere. The cells were washed with PBS, replaced with fresh full medium and further incubated for 44 h. The transfection experiments were done in triplicate. A transfection formulation with L-pEI prepared at an optimal N/P ratio of 10/1 or with Lipofectamine 2000 (1.5 μL +1 μg siRNA), were applied as positive controls. The cells treated with PBS were used as negative control. The luciferase protein expression was analyzed using luciferase reporter gene assay (Promega). Briefly, after 44 h, the cells were washed with 100 μL cold PBS and lysed with 50 μL lysis buffer. Next, 20 μL cell lysate was mixed with 50 μL luciferase assay reagent (Promega) with a microinjector, and after 2 s, luminescence was measured for 10 s using a FLUOstar OPTIMA microplate, equipped with a luminescence light guide (BMG LabTech, Germany). Polyplexes based on the commercially available transfection agents L-PEI and Lipofectamine 2000 were used as positive controls. The gene silencing results were corrected for cytotoxicity, and the results are expressed as relative luciferase expression compared to untreated cells (Varkouhi et al., 2012).

2.7. Cell viability assay

The cytotoxicity of the different polymers or the siRNA polyplexes was determined by the AlamarBlue assay (Invitrogen). In brief, Skov3-luc cells were incubated with polymer solutions (concentrations ranging from 2 to 100 μg/mL) or siRNA polyplexes (concentrations ranging from 100 to 200 nM siRNA, at N/P ratio of 8 or 16) for 4 h in the absence or presence of serum, and subsequently the medium was replaced by fresh full medium. After 40 h, the medium was removed and the cells were washed twice with PBS buffer and incubated with freshly made 100 μL1 × Alamar Blue-DMEM medium (i.e.10-fold diluted AlamarBlue in DMEM full medium) for 4 h. Thereafter, 80 μL of medium from the different wells (including 1 × AlamarBlue-DMEM medium as a blank) were transferred into a 96-well plate and the UV optical density (OD) was measured using a plate reader (Spectrostar, BMG, Germany) at 570 nm and 630 nm, respectively. Cell viability was calculated as follows:

$$\text{Cell viability } (\%) = (OD_{sample} - OD_0)/OD_{control} - OD_0) \times 100,$$

where OD_{sample}, $OD_{control}$, and OD_0 are the OD values of the medium of transfected cells, the medium of untreated cells, and 1 × AlamarBlue-DMEM medium, respectively. The value for untreated cells was taken as 100% cell viability.

2.8. Cellular uptake of polyplexes

Skov3-luc cells were seeded into a 96-well plate (10,000 cells/well) and incubated for 24 h with the growth medium, DY-547 labeled siRNA was used to prepare polyplexes. Next, the cells were incubated with the polyplexes (final siRNA concentration was 100 nM, N/P ratio of 16) for 4 h at 37 °C with or without serum. The cells were then washed twice with ice-cold PBS. To quench the fluorescence of polyplexes associated with the cell membrane, the cells were incubated with 0.4% trypan blue-containing PBS for 5 min and washed with PBS (Naik et al., 2011). After trypsinization, the cells were collected and suspended in PBS. Cellular uptake of siRNA polyplexes was examined by a flow cytometry with a help of Canto II (BD, USA).

2.9. Confocal laser scanning microscopy studies

Skov3-luc cells were seeded into 96-well μClear® black plates (7000 cells/well) and incubated for 24 h. Then, the medium was replaced with fresh medium with or without serum. Polyplexes with DY-547 labeled siRNA (final siRNA concentration was 100 nM) were added and the cells were subsequently incubated for another 2 or 4 h at 37 °C. Before the observation, the medium was replaced with fresh full medium containing Hoechst33342 and Wheat Germ Agglutinin-Oregon Green® conjugate (Molecular probes, Oregon, USA) for staining the nuclei and cell membranes, respectively (incubation at 37 °C for 5 min). After washing with PBS, the cells were fixed with 2% formaldehyde (w/v) (diluted form Pierce™ 16% formaldehyde (w/v) with PBS). CLSM images were recorded using Yokogawa CV7000S imager (Yokogawa group, Tokyo, Japan) equipped with a 60× water immersion objective at excitation wavelength of 405, 488 and 561 nm for Hoechst33342, Oregon Green® 488 and DY-547, respectively.

2.10. Statistical analysis

p Values were determined by Student's test with two-tailed distribution performed with the software GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California). *p* Values <0.05 were considered as statistically significant.

3. Results and discussion

3.1. Physicochemical characteristics of polymer/siRNA polyplexes

Three cationic PVTC-based polymers were used to study their ability to condense siRNA into nano-sized polyplexes, namely one homopolymer PVTC and two block copolymers composed of PVTC and POEGMA (PP2 and PP4). The polymer chemical structures are shown in Fig. 1. Compared to plasmid DNA (several kilo bp), siRNA only consists of two short annealed oligonucleotides of 20–23 bp,

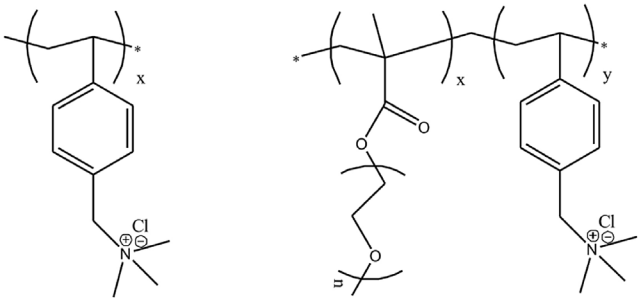

Fig. 1. Structures of poly(vinyl benzyl trimethyl ammonium chloride) (left, PVTC x = 224) and poly[oligo(ethylene glycol) methacrylate]-b-poly(vinyl benzyl trimethyl ammonium chloride) block copolymer (right, PP2 x = 38 and y = 24, PP4 x = 38 and y = 87).

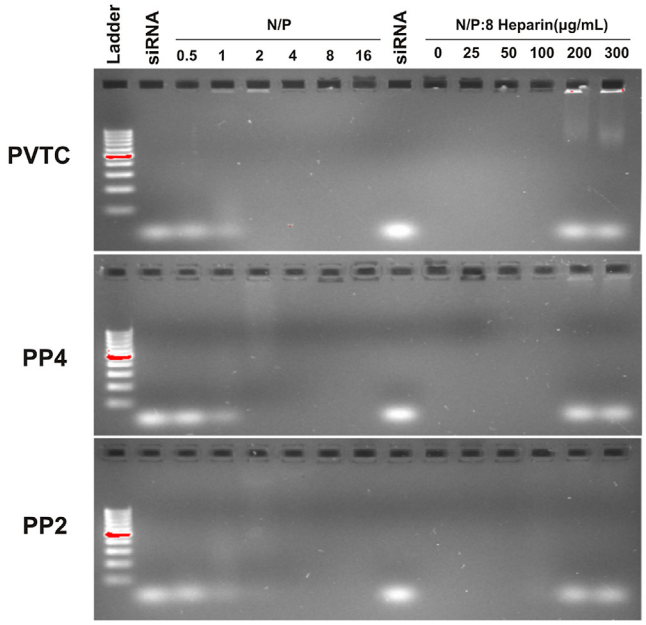

Fig. 2. Agarose gel retardation assay of the PVTC/siRNA (up), PP4/siRNA (middle) and PP2/siRNA (bottom) polyplexes at different N/P ratios. To study heparin induced polyplex destabilization, siRNA polyplexes of N/P 8 were incubated with heparin (different concentrations) for 10 mins in 10 mM HEPES buffer (pH 7.4, containing 150 mM NaCl).

and thus has less electrostatic interactions with polycations. As a result, it is more difficult for cationic polymers to condense siRNA and form stable particles with this nucleic acid based drug (Liu et al., 2016; Scholz and Wagner, 2012).

With increase of N/P ratio, because of the excess of cationic amines, all three polyplexes showed positive zeta potentials (Fig. S2).The binding ability of PVTC-based polymers with siRNA was evaluated by a gel retardation assay. Fig. 2 shows that all polymers exhibited good siRNA binding at N/P ratio of 4 and above, where complete siRNA retardation was observed. Studies have shown that competition between biologically polyanionic molecules (such as proteins and polysaccharides) and cationic polyplexes may occur under physiological conditions. This may cause the release of siRNA from the polyplexes and also can result in shorter circulation kinetics of the nucleic acid and lower transfection efficiency (Dakwar et al., 2014; Mao et al., 2006; Sato et al., 2007). The strongly negatively charged polysaccharide heparin was added to the siRNA polyplexes at increasing concentrations to investigate the particle stability (Fig. 2). In this case, polyplexes prepared at N/P ratio of 8 was used for this study because Firgure 2 shows that the polymers fully complex siRNA at this ratio. We found that siRNA polyplexes based on all three polymers at N/P ratio of 8 still complexed siRNA strongly at heparin concentration up to 100 μg/mL. At a heparin concentration of 200 μg/mL and higher, siRNA was completely released from PP4/PP2 polyplexes, whereas only part of siRNA released from the PVTC/siRNA polyplexes. This heparin competition study demonstrates that strong electrostatic interactions occur between the quaternary amine groups of the polymers and the phosphate groups of siRNA, which is accordance with previous studies (Delisavva et al., 2013; Varkouhi et al., 2012).

To further study the siRNA complexation ability of the different polymers, DLS and zeta-potential measurements were carried out with siRNA polyplexes. As shown in Table 2, all three polymers were able to bind to siRNA to yield positively charged polyplexes with an average diameter smaller than 25 nm (PDI < 0.3). The PVTC polymer, having the longest polycation segment, formed polyplexes with siRNA of around 20 nm, which was close to the size visualized by TEM (Fig. S1). Interestingly, the PP4 and PP2 polymers with shorter polycation chains formed particles of smaller diameter, 14 nm and 9 nm, respectively. The smaller size of the PP2 and PP4 polyplexes as compared those based on PVTC at the same N/P ratio could be explained by the presence of PEG chain; similar results have also been observed in previous studies (Kim et al., 2012; Lin et al., 2008). Such small nanosized siRNA polyplexes (10–20 nm) were also found in previous studies of polyplexes based a block copolymer of poly(ethylene glycol)-b-poly(L-lysine) (PEG-LL) (Christie et al., 2012; Derouchey et al., 2008; Florinas et al., 2016; Hayashi et al., 2016). siRNA polyplexes with diameter around 10 nm were considered as monomolecular assembly of oppositely charged siRNA and PLL, i.e., a particle composed of a single siRNA molecule coated with PLL-PEG via electrostatic interactions. We hypothesize that the polymers used in this study had similar assembling behavior as PEG-PLL, where each polyplex particle contained one molecule of siRNA.

With increase of N/P ratio, because of the excess of cationic amines, all three polyplexes showed slightly increase of surface charges (Fig. S2). At N/P ratio of 16, the zeta potential of siRNA polyplexes prepared by homopolymer PVTC was the highest (27.6 mV), whereas the PP4 and PP2 siRNA polyplexes have lower zeta potential (19.7 and 9.2 mV, respectively)which can be attributed to the shielding effect of the neutral and hydrophilic POEGMA moieties (Uezguen et al., 2010; Verbaan et al., 2004; Zoetebier et al., 2016).

An effective siRNA delivery system should retain its colloidal stability under physiological conditions. Therefore, we investigated the stability of the polyplexes (N/P = 16) in 140 mM NaCl/10 mM

Table 2
Size and zeta potential of siRNA polyplexes.[a]

Polyplexes	Size (nm) ± S.D.	Zeta potential (mV) ± S.D.	PDI
PVTC	19 ± 2	27.6 ± 1.4	0.24
PP4	14 ± 1	19.7 ± 2.1	0.20
PP2	9 ± 1	9.2 ± 3.2	0.27

[a] Prepared at N/P = 16 and a siRNA concentration of 10 μg/mL in 10 mM Hepes buffer, pH 7.4.

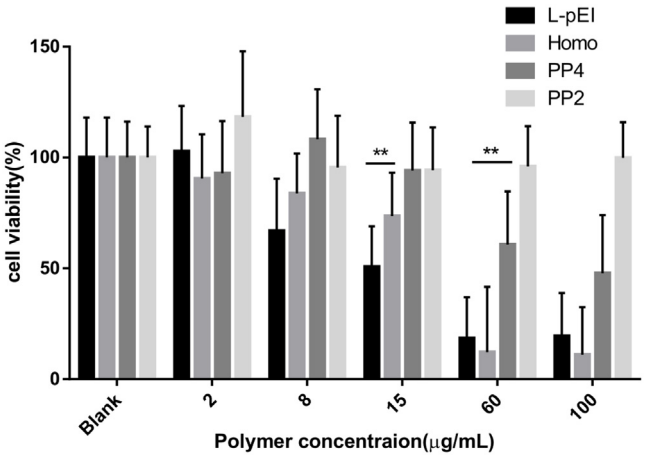

Fig. 3. Viability of Skov3-luc cells after incubation with polymers or L-pEI in serum free medium for 4 h at 37 °C. All values are given as the mean ± SD (n = 3). Representative results from one of the three experiments are shown. **p < 0.01.

HEPES buffer (Fig. S3). DLS measurements showed that the size of the three different polyplexes remained constant after incubation in this buffer for 24 h, which can be ascribed to electrostatic repulsion between the nanoparticles due to their relatively high zeta potential for the PVTC/siRNA polyplexes and steric stabilization of the particles due to the POEGMA blocks of the PP4/siRNA and PP2/siRNA polyplexes.

3.2. In vitro *cytocompatibility and gene silencing activity of the polyplexes*

The cytotoxicity of the PVTC-based polymers was assessed in the human ovarian adenocarcinoma cell line (Skov3-luc) in the absence of serum using AlamarBlue assay (Fig. 3). In this experiment, L-pEI was used as control polycation. L-pEI was significantly more cytotoxic than the PVTC-based polymer at a polymer concentration of 15 μg/mL and above. The 50% inhibitory concentration (IC_{50}) for the homopolymer was two times higher than that of PEI (Table S1). It is known that the cytotoxicity of polycations is a function resulting from the polyamine nature (i.e., primary, secondary, tertiary, and quaternary amino groups) rather than its charge density (Fischer et al., 2003; Kim et al., 2016). Previous studies have shown that although polymers with quaternary amine groups have a higher charge density, they exhibited lower toxicity compared to tertiary polyamines due to their lower cell membrane disruptive activity (Lee et al., 2003; Patil et al., 2008; Tamura et al., 2010). The IC_{50} values of PP4 and PP2 polymers were substantially higher compared to the PVTC homopolymer (92 μg/mL vs 32 μg/mL), which may be due to the hydrophilic blocks (POEGMA) but also because of the shorter amine blocks (Fischer et al., 1999). For the cell transfection experiments we used a maximum polymer concentration of 15.6 μg/mL, which is far below their IC_{50} values.

The luciferase gene silencing (RNAi) activities of the siRNA polyplexes in Skov3-luc cells were examined as a function of the N/P ratio and siRNA concentration both in the absence (Fig. 4A) and presence of serum (Fig. 4C). At N/P 8 and siRNA concentration of 100 nM, PVTC and PP4 polyplexes showed a knock down of around 80% of luciferase expression, which was comparable to the efficiency of the lipofectamine formulation and significantly higher than that of the L-pEI/siRNA polyplexes (~35%), as shown in Fig. 4A. On the other hand, PP2/siRNA showed negligible gene silencing activity at N/P ratio of 8. Incubation of the cells with the three polyplexes prepared at N/P ratio of 16 resulted in similar gene silencing effects as observed for polyplexes of N/P of 8. Additionally, increasing the polyplexes dose to 200 nM siRNA did not increase the knock down efficiency. However, increasing the N/P ratio to 16 and the dose of the polyplexes to 200 nM siRNA, led to a drop in cell viability, which can be explained by the higher concentrations of polymer to which the cells were exposed (Fig. 4B). It should be noted that at N/P of 8 the cytotoxicity of polyplexes was mild (~80% cell viability), being even lower than that of the lipofectamine formulation (~45% cell viability). The higher cell viability of PEGylated PP4/PP2 polymers compared to PVTC at higher N/P ratio and siRNA dose demonstrates that the POEGMA corona could effectively reduce the cytotoxicity by

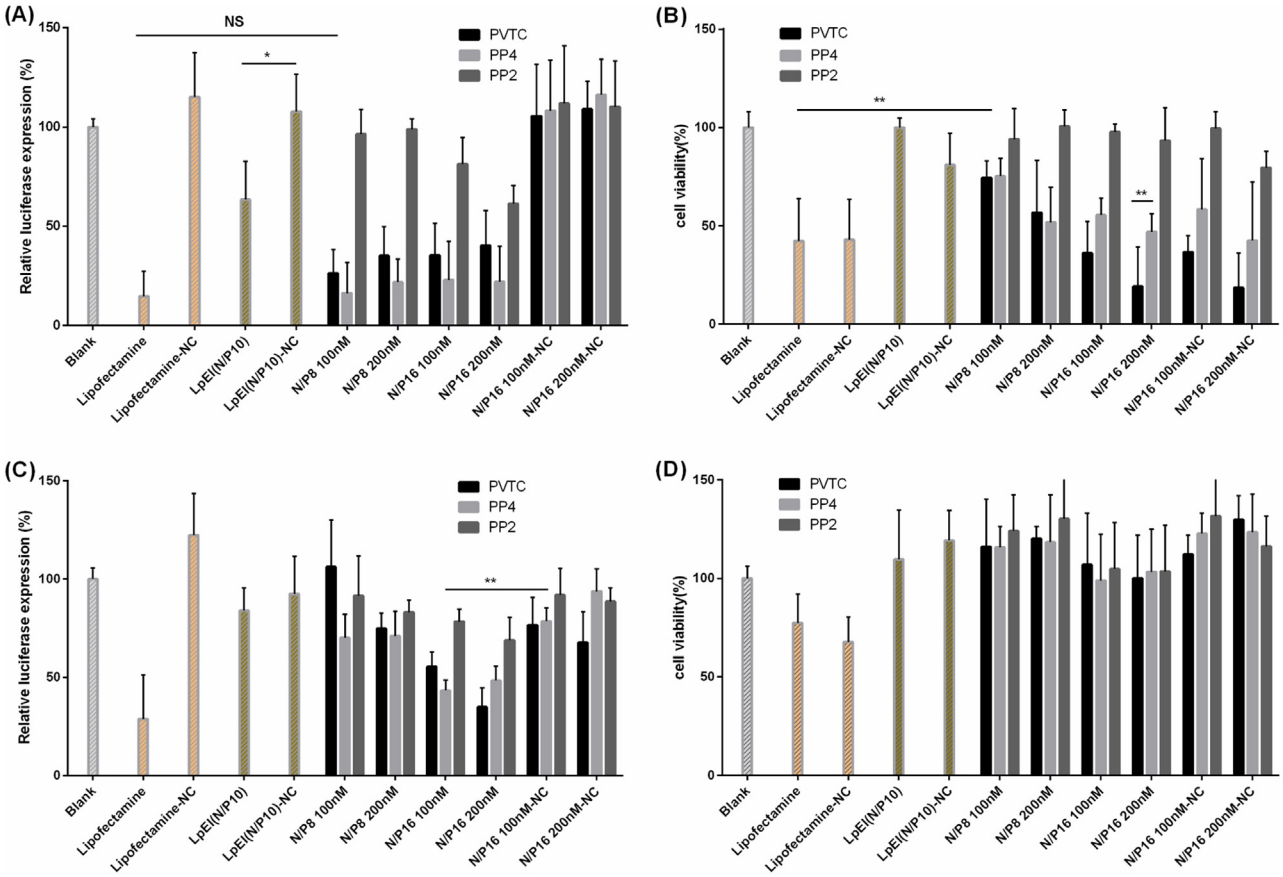

Fig. 4. Luciferase gene expression after incubation of Skov3-luc cells with siRNA polyplexes in the absence (A) or presence (C) of serum. Cell viability was measured by AlamarBlue assay after incubation of Skov3-luc cells with siRNA complexes in the absence (B) or presence (D) of serum. siRNA concentration was 100 or 200 nM. NC: Negative control siRNA. Representative results from one of the three experiments are shown. All values are given as the mean ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

shielding the surface charge of polyplexes. Moreover, comparison of the silencing effects between anti-luciferase siRNA and negative control siRNA polyplexes (N/P 16, Fig. 4A) supports the fact that the reduced luminescence observed for the polyplex formulations was due to the specific inhibition of luciferase expression and not because of their cytotoxicity.

The transfection activity of the polyplexes was also investigated in medium with serum (Fig. 4C). Fig. 4C shows that at N/P 8 and a siRNA dose of 200 nM, the luciferase knock down efficiency was substantially decreased to 20–30%. At N/P 16 and siRNA dose of 200 nM the PVTC and PP4 based polyplexes led to about 60 and 50% gene silencing, respectively, which was slightly lower than in the absence of serum (70% and 75% respectively). The reduction in transfection activity of the polyplexes in the presence of serum might be explained by the adsorption of negatively charged serum proteins onto the polyplexes surface resulting in polyplexes destabilization and/or reduced cellular uptake due to the polyplexes charge reversal (De Wolf et al., 2005; Gosselin et al., 2001). The cell viability was maintained close to 100% for all polyplex formulations, and the cytotoxicity of lipofectamine formulation was reduced as compared to the serum-free conditions (Fig. 4D).

3.3. Cellular uptake of siRNA polyplexes studied by flow cytometry and CLSM

The cellular uptake of DY-547 labeled siRNA polyplexes by Skov-3-luc cells was studied using flow cytometry and confocal laser scanning microscopy (CLSM). Fig. 5 presents the percentage of DY-547 positive cells as well as mean fluorescence intensity (MFI) of the cell population upon incubation with different formulations in the absence or presence of serum. As shown in Fig. 5A&B, the polyplexes based on PP4 and PP2 showed lower cellular uptake compared to PVTC/siRNA polyplexes, presumably due to the lower zeta potential as a result of the POEGMA corona. Moreover, the PP4, PVTC and lipofectamine formulations showed much higher cell uptake than the polyplexes based on L-pEI and

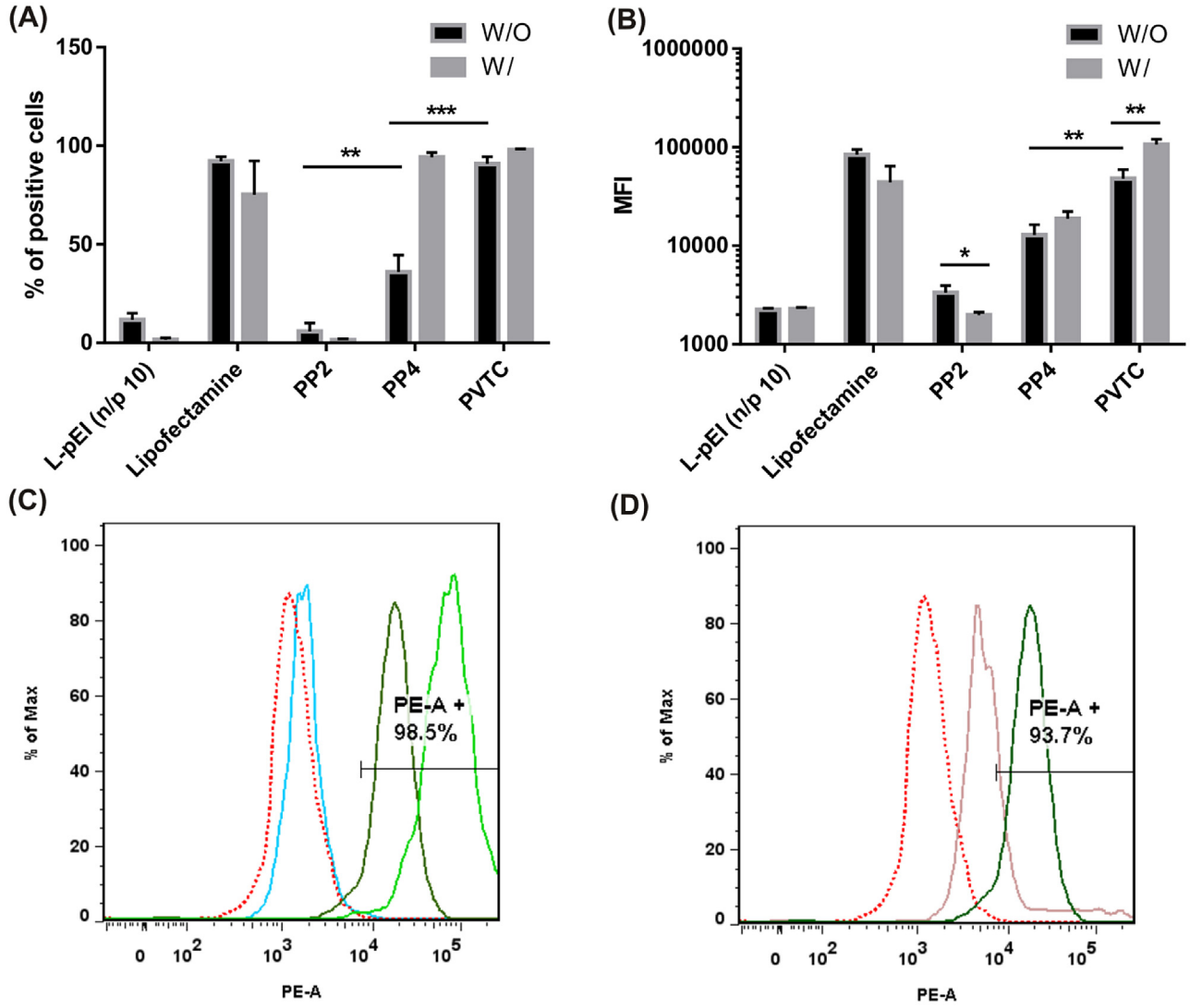

Fig. 5. Cellular uptake of DY-547 labeled siRNA polyplexes (N/P = 16) by Skov3-luc cells in the absence (W/O) or presence (W/) of serum; L-pEI 25 kDa and lipofectamine based formulations were used as controls. (A) Percentage of the DY-547 positive cells. (B) Mean fluorescence intensity of the cell population. Data represent the means ± SD (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (C) Fluorescence histogram of Skov3-luc cells incubated with the different polyplexes in the presence of serum: untreated cells (red dots), PP2/siRNA (blue), PP4/siRNA (dark-green) and PVTC/siRNA (green). (D) Fluorescence histogram of Skov3-luc cells with PP4/siRNA polyplexes in the absence (pink) or presence (dark green) of serum; untreated cells were used as a blank (red dots). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

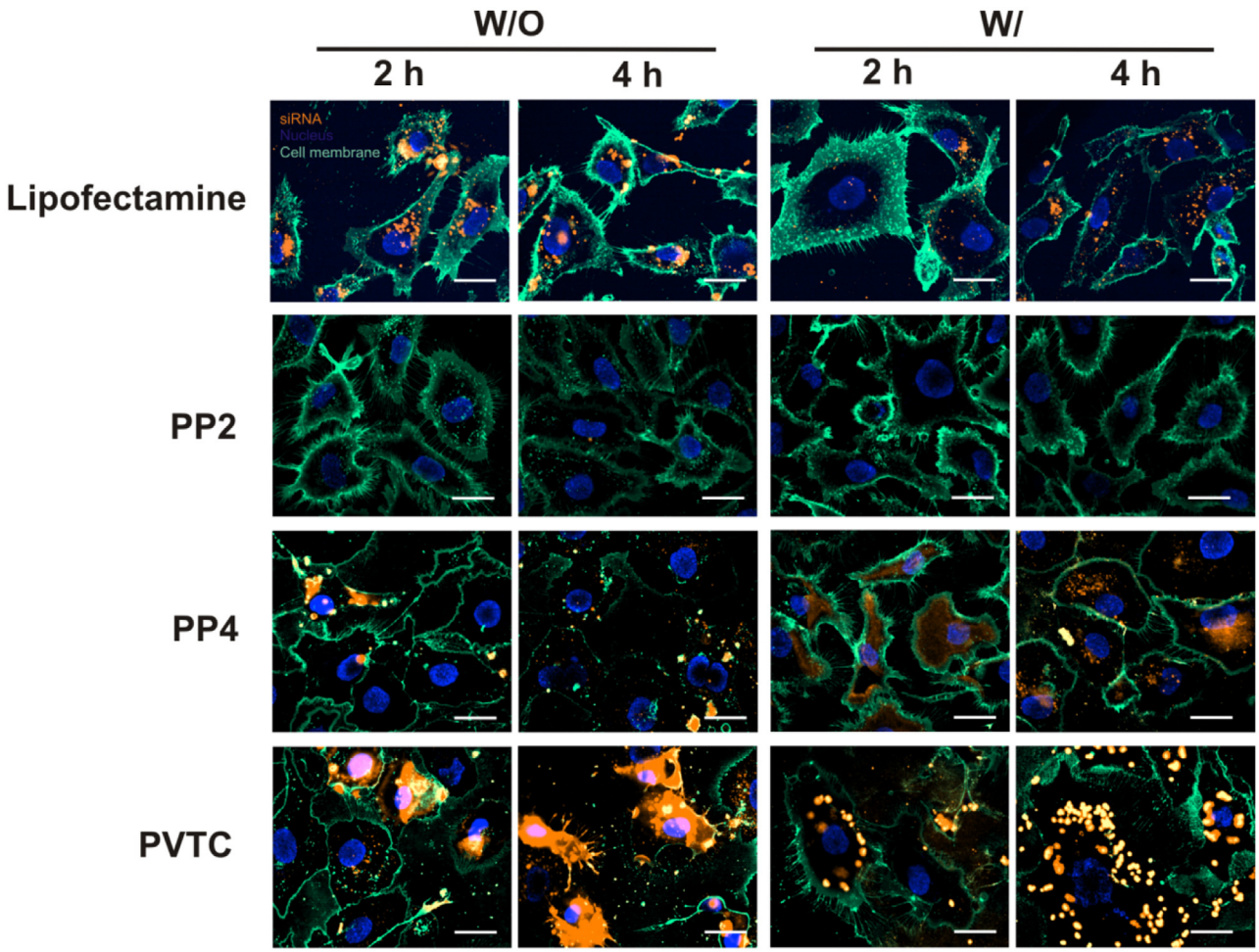

Fig. 6. Confocal microscopy images of Skov-3-luc cells after 4 h incubation with different polyplexes in the absence (Left, W/O) and presence (Right, W/) of serum. All images are overlays of the fluorescent signals from DY547-siRNA (orange), cell membrane Wheat Germ Agglutinin-Alexa Fluor® 488 (green), and cell nucleus Hoechst33342 (blue). Bars indicate 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

PP2. Additionally, a higher cellular uptake was observed when the cells were incubated with PVTC and PP4 based polyplexes in the presence of serum (Fig. 5D), which might be caused by the positive charge of the PVTC/siRNA (27.6 mV) and PP4/siRNA polyplexes (19.7 mV). For positively charged particles the presence of serum in the incubation medium has shown to increase their cellular uptake (Dakwar et al., 2015; Naik et al., 2011; Yin et al., 2011). In contrast, the cellular uptake level of DY547-siRNA for PP2 and L-pEI polyplexes was negligible in the presence of serum, most likely due to the low stability of the complexes (Fig. 5A&B). Therefore, the high gene silencing activities of the PVTC/siRNA and PP4/siRNA complexes can be ascribed to their high cellular uptake.

Examination of polyplexes uptake by Skov-3-luc cells with confocal laser scanning microscopy confirmed the flow cytometric analysis (Fig. 6). As expected, PP2/siRNA complexes showed the lowest degree of cell internalization. The PP4/siRNA polyplexes displayed time-dependent uptake in both serum-free and serum-containing media. Moreover, the serum proteins had an obvious influence on the uptake profile of PP4/siRNA as the subcellular localization of labeled siRNA significantly differed for the studied conditions (with or without serum). The more diffused cytosolic signal from the cells incubated with the polyplexes in presence of serum might indicate higher degree of siRNA release from the polyplexes after cellular internalization. Interestingly, the uptake profile of PVTC/siRNA polyplexes was also highly affected by the presence of serum. In serum-free medium PVTC/siRNA polyplexes displayed a more diffusive cytosolic signal in contrast to round micrometer-sized vesicles localized in the perinuclear space observed for cells in a serum-containing medium.

Taken together, the PVTC-based polymers are capable of silencing luciferase expression both in the absence and presence of serum. The PP4 polymer is the most promising candidate since it formed small polyplexes (~14 nm) and displayed lower cytotoxicity with good gene silencing knock down efficiency in the presence of serum (Fig. 4). It has been demonstrated in many studies that the particle size plays an important role for the cellular uptake and subsequent trafficking of polyplexes (Albanese et al., 2012; Cabral et al., 2011; Jiang et al., 2008; Nuhn et al., 2014). For siRNA or DNA delivery systems, endosomal escape is considered to be a major bottleneck in the transfection process. Generally, siRNA polyplexes with a size of 80–200 nm are internalized by clathrin-mediated endocytosis, and those particles would accumulate in the late endosomes associated with oligonucleotide degradation (Lachelt and Wagner, 2015; Whitehead et al., 2009). Recent studies have shown that small-sized particles (<50 nm) are preferentially internalized by caveolin-mediated endocytosis to end up in 50 nm sized caveolae, this pathway is known to avoid vesicle fusion with lysosomes and subsequent siRNA degradation (Harris

et al., 2002; Nuhn et al., 2014; Sahay et al., 2010). The PVTC-based polymers in this study complexed siRNA to particles with sizes less than 25 nm (Table 2). Therefore, we expect that these particles are internalized by cells through caveolin-mediated endocytosis, avoiding endolysosomal uptake pathways which might explain why PVTC-based polyplexes possessed such high gene silencing efficiencies. Nevertheless, a detailed investigation to prove this assumption will be a subject of future studies.

4. Conclusion

In this study, we evaluated a series of PVTC-based polymers that are capable of delivering intact siRNA intracellularly resulting in gene silencing. The quaternary amine groups of PVTC enable the polymer to form colloidally stable, small nano-sized particles (<25 nm) with siRNA. Polyplexes based on PP4 block copolymer with a hydrophilic and uncharged POEGMA moiety exhibit excellent gene silencing efficiency without compromising the cell viability both in the presence and the absence of serum proteins. The results presented in this paper demonstrate that the PP4/siRNA polyplexes formulations are attractive candidates for siRNA therapy by means of I.V. administration, as they are large enough to escape rapid renal clearance (~10 nm cutoff) but small enough to penetrate into the tissues, e.g. solid tumors.

Acknowledgment

The research was partially supported by China Scholarship Council.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2017.03.036.

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