International Journal of Pharmaceutics 411 (2011) 197–205

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Synthesis of a new potential biodegradable disulfide containing poly(ethylene imine)–poly(ethylene glycol) copolymer cross-linked with click cluster for gene delivery

Nan Zhao, Susanne Roesler, Thomas Kissel*

Department of Pharmaceutics and Biopharmacy, Philipps-University of Marburg, Ketzerbach 63, D-35032 Marburg, Germany

ARTICLE INFO

Article history:
Received 14 January 2011
Received in revised form 13 March 2011
Accepted 15 March 2011
Available online 23 March 2011

Keywords:
PEG–PEI copolymer
Biodegradable
Disulfide
Click chemistry

ABSTRACT

Poly(ethylene glycol)-grafted-polyethylenimine (PEG–PEI) are promising non-viral gene delivery systems. Herein, we aimed to synthesize a biodegradable disulfide containing PEGylated PEI to attempt to reduce its cytotoxicity and enhance the gene transfer activity. Using click chemistry, low Mw PEI (br. 2 kDa) and short chain length PEG (tetraethylene glycol, TEG) were cross-linked to a high Mw PEG–PEI copolymer (~22 kDa). The chemical structure of the copolymer was characterized using ^{1}H NMR and GPC. The degradation behavior was investigated under *in vitro* conditions in the presence of 1,4-dithiothreitol (DTT). The gel retardation assay, dynamic light scattering and atomic force microscopy showed good DNA condensation ability by forming polyplexes with small particle size and positive zeta potential. In particular, MTT assay indicated that this PEG–PEI polymer is about 22-fold less toxic than PEI 25k and only 2-fold more toxic than PEI 2k in L929 cell line. After coupling of small PEG chains and cross-linking by disulfide bridges, the transfection efficiency is increased approximately 6-fold in comparison to PEI 2k and still reaches approximately 17% of PEI 25k. Hence, this click cluster cross-linked disulfide containing PEG–PEI copolymer could be an attractive cationic polymer for non-viral gene delivery.

1. Introduction

Gene therapy has been considered as a promising approach for the treatment of acquired or inherited diseases that are currently incurable. Since therapy with viral gene vectors has been limited by several drawbacks such as random DNA insertion, risk of replication, and possible host immunogenicity, non-viral vectors have been gaining significant recognition over the last decade because of their non-immunogenicity and safety. Among those, poly(ethylene imine) (PEI) based cationic polymers are some of the most potential cationic polymers, which have been shown to have a high ability of DNA condensation and high transfection efficiency due to their high cationic charge potential (Lungwitz et al., 2005; Neu et al., 2005; Gao et al., 2007; Luten et al., 2008).

However, transfection efficiency as well as cytotoxicity of PEI is strongly correlated to its molecular weight. Higher molecular weights lead to higher transfection efficiency but increased cytotoxicity, whereas low molecular weight PEI less than 2k displays less toxicity but almost no transfection on the contrary (Godbey et al., 1999a; Fischer et al., 1999). A commonly used strategy to overcome this problem is to synthesize highly branched HMW PEI consisting of LMW PEI oligomers and possible degradable cross-links. Lee et al. cross-linked LMW PEI using DSP (dithiobis(succinimidylpropionate)) and DTBP (dimethyl-3,3′-dithiobis(propion- imidate)) to efficient gene transfer with half of the efficiency but reduced toxicity compared to PEI 25k (Gosselin et al., 2001). Forrest et al. synthesized gene vectors with mediate transfection efficiency and low toxicity via cross-linking PEI 800 Da with ester bonds using diacrylates (Forrest et al., 2003).

Another drawback of PEI correlated with its high charge density is its aggregation and short circulation time in the bloodstream *in vivo*. For resolving this problem PEGylation is a popular strategy for shielding of PEI/DNA polyplexes. PEG is a widely used biocompatible shielding reagent and can additionally improve the water solubility (Kichler et al., 2002; Tang et al., 2003; Ogris et al., 1999). Among those, AB and ABA copolymers consisting of high Mw PEG and PEI have already shown efficient DNA condensation and improved pharmacokinetic properties, but the limitation is the lack of degradability under *in vivo* condition (Luten et al., 2008; Neu et al., 2007a).

Cross-linking with biodegradable disulfide bonds is an attractive strategy, since disulfide bonds are cleavable in the reductive environment of cytoplasm. Disulfide cross-linked PEI and disulfide

* Corresponding author. Tel.: +49 6421 2825881; fax: +49 6421 2827016.
E-mail address: kissel@staff.uni-marburg.de (T. Kissel).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.03.038

cross-linked PEG were reported in recent years (Lee et al., 2005, 2007; Peng et al., 2009). Disulfide cross-linked PEI (22 kDa, 25 kDa) and PEG (5–40 kDa) connecting with ligand transferrin (Tf) was as well synthesized and showed comparable transfection efficiency as Tf–PEI 25k (Kursa et al., 2003). Therefore, a fully degradable disulfide containing PEG–PEI copolymer consisting of LMW PEI and LMW PEG was interesting to synthesize and test.

Click chemistry, as a useful tool in linker chemistry for application in pharmaceutical research (Hein et al., 2008; Tron et al., 2008; Dedola et al., 2007), has offered a series of advantages, such as its reactivity under mild conditions, formation of regioselective products with good yield and easy purification. It was reported that click chemistry is one of the simplest and most efficient polymer chemistry to join any two homopolymer blocks together to form block copolymers, which was difficult to be realized using normal organic chemical methods (Hein et al., 2008). Among those copper catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes is the most widely used. The resulting 1,2,3-triazole ring possesses a number of desirable features reported in the medicinal chemistry (Tron et al., 2008) and is for example stable under standard physiological conditions, which suits well for gene delivery system. For this reason we finally chose Huisgen 1,3-dipolar cycloaddition to conjugate PEG- and PEI-block after introducing disulfide bonds in PEG chains. Disulfide-containing cross-linked polyethylenimines (PEI-SS-CLs, PEI-SS-HP) synthesized via click chemistry were reported recently (Liu et al., 2010; Jiang et al., 2011), showing less cytotoxicity and superior transfection activity as compared to PEI 25 kDa. However, no papers using click chemistry for disulfide-containing PEG–PEI cross-linking have been published so far.

According to all these considerations we aimed to synthesize a biodegradable PEG–PEI copolymer building from LMW PEI and disulfide cross-linked LMW PEG through coupling with click chemistry. Branched PEI 2 kDa and a short chain PEG (TEG) were selected in hopes that the copolymer can be totally converted into biodegradable small breakdown products after cleavage of disulfide bonds in the lysosomal compartment. The synthetic route of the azides modified, disulfide containing PEG linker consisted of several steps which were nonetheless easily carried out and purified. The PEG–PEI copolymer was obtained by means of coupling PEG linker and alkynes modified PEI using Huisgen 1,3 dipolar cycloaddition and characterized with ^{1}H NMR and GPC. In addition, the degradation behavior and the DNA condensation ability of the copolymer, as well as its transfection efficiency and cytotoxicity in cells were also investigated.

2. Materials and methods

2.1. Materials

Branched poly(ethylene imine) with a molecular weight of 2 kDa, 5 kDa and 25 kDa were gifts from BASF, Ludwigshafen, Germany. Tetraethylene glycol was purchased from Aldrich. pCMV-luc Plasmid was purchased from Plasmid Factory (Bielefeld, Germany). Herring testes DNA (Sigma, Taufkirchen, Germany) was used for light scattering experiments, DNA condensation assay and gel retardation assay. Plasmid DNA (pklac1: 9091 bp) used for AFM measurement was a product of NEB (New England Biolabs, UK). L929 cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in DMEM low glucose (PAA, Cölbe, Germany) supplemented with 150 mM glutamine containing 10% fetal calf serum (FCS, Cytogen, Sinn, Germany) and incubated in 37 °C and 8.5% CO_2 atmosphere. All other chemicals and solvents were used as received without further purification.

2.2. Synthesis of 2-{2-[2-(2-hydroxyethoxy)ethoxy]ethoxy}ethyl 4-methylbenzene-sulfonate (HO-TEG-OTs) (1)

Tetraethylene glycol (50 mL, 0.290 mol, 2 equiv.) was dissolved in 120 mL of anhydrous dichloromethane. Under a protective inert gas 30.3 mL of triethylamine (0.217 mol, 1.5 equiv.) was added and the resulting mixture was cooled to 0 °C. After *para*-toluenesulfonyl chloride (27.92 g, 0.145 mol, 1 equiv.) was added, the mixture solution was allowed to warm up to room temperature and stirred for 24 h. After filtration the clear solution was poured into water (150 mL). The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with water and brine and dried over $MgSO_4$. After evaporation of the solvent under reduced pressure the product **1** was obtained as yellow oil (31.8 g, 63% yield with reference to tetraethylene glycol). ^{1}H NMR ($CDCl_3$) δ ppm: 2.44 (*s*, 3H, $-CH_3$); 3.53–3.75 (*m*, 14H, $-CH_2-O-$); 4.16 (*t*, 2H, $-CH_2-$OTs); 7.32 (*d*, 2H, –CHAr–); 7.78 (*d*, 2H, –CHAr–). ^{13}C NMR ($CDCl_3$) δ ppm: 21.7 ($-CH_3$); 61.9 ($-CH_2-$OH); 68.9, 69.3, 70.5, 70.6, 70.8, 70.9 ($-CH_2-CH_2-O-$); 72.6 ($-CH_2-CH_2-$OH); 128.1, 129.9 ($-CH_{Ar}-$); 133.2 ($-C_{Ar}-SO_2-$); 144.9 ($-C_{Ar}-CH_3$).

2.3. Synthesis of 2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethanol (HO-TEG-N_3) (2)

Sodium azide (9.3 g, 143.0 mmol, 5 equiv.) was added to monotosylate **1** (10.0 g, 28.8 mmol, 1 equiv.) in dry DMF (100 mL). The reaction mixture was stirred at 60 °C for 16 h. The precipitate was filtered and the solvent was removed under vacuum. The residue was placed in water and extracted with CH_2Cl_2 (3 × 100 mL). The combined organic layers were washed with water and brine and dried over Na_2SO_4. After evaporation of the solvent under reduced pressure the product **2** (5.4 g, 85%) was obtained as yellow oil. ^{1}H NMR ($CDCl_3$) δ ppm: 3.35 (*t*, 2H, $-CH_2-N_3$); 3.57 (*t*, 2H,$-CH_2-CH_2-N_3$); 3.60–3.74 (*m*, 14H, $-CH_2-CH_2-O-$). ^{13}C NMR ($CDCl_3$) δ ppm: 50.7 ($-CH_2-N_3$); 61.8 ($-CH_2-$OH); 70.1, 70.4, 70.6, 70.7, 70.8 ($-CH_2-CH_2-O-$ and–$CH_2-CH_2-N_3$); 72.5 ($-CH_2-CH_2-$OH).

2.4. Synthesis of (2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl 4-methylbenzene -sulfonate) (TsO-TEG-N_3) (3)

Triethylamine (5.1 mL, 36.3 mmol, 1.5 equiv.) was added to a solution of **2** (5.3 g, 24.2 mmol, 1 equiv.) in CH_2Cl_2 (100 mL). The reaction mixture was cooled down to 0 °C and *p*-toluenesulfonyl chloride (5.6 g, 29.0 mmol, 1.2 equiv.) was added and stirred for 1 h. The reaction mixture was then allowed to warm to room temperature and stirred for 16 h. $NaHCO_3$, water and brine were used to wash the reaction mixture one after another. Drying of the organic layer over Na_2SO_4 and evaporation of the solvent under reduced pressure afforded **3** (7.65 g, 84.6% yield) as brown oil. ^{1}H NMR ($CDCl_3$) δ ppm: 2.44 (*s*, 3H, $-CH_3$); 3.37 (*t*, 2H, $-CH_2-N_3$); 3.61–3.71 (*m*, 12H, $-CH_2-O-$); 4.15 (*t*, 2H, $-CH_2-$OTs); 7.32 (*d*, 2H, –CHAr–); 7.78 (*d*, 2H, –CHAr–). ^{13}C NMR ($CDCl_3$) δ ppm: 21.7 ($-CH_3$); 50.8 ($-CH_2-N_3$); 68.8, 69.4, 70.2, 70.7, 70.8, 70.9 ($-CH_2-CH_2-O-$ and $-CH_2-CH_2-N_3$); 128.1, 129.9 ($-CH_{Ar}-$); 133.3 ($-C_{Ar}-SO_2-$); 144.9 ($-C_{Ar}-CH_3$).

2.5. Synthesis of S-2-(2-(2-(2-azidoethoxy)ethoxy)ethoxy)ethyl ethanethioate (N_3-TEG-SAc) (4)

Potassium thioacetate (4.68 g, 40.98 mmol, 2 equiv.) was added to a solution of **3** (7.65 g, 20.49 mmol, 1 equiv.) in acetonitrile (100 mL). The reaction mixture was heated to 60 °C whilst stirring for 16 h. After filtration of the precipitate the solvent was evaporated. The residue was placed in water and extracted with

CH_2Cl_2. Drying of the organic layer over Na_2SO_4, evaporation of the solvent under reduced pressure and purification using column chromatography (diethyl ether/pentane, 4:6 to 6:4) afforded **4** (1.91 g, 33% yield). ^{1}H NMR ($CDCl_3$) δ ppm: 2.33 (*s*, 3H, $-CH_3$); 3.09 (*t*, 2H, $-CH_2-S-$); 3.39 (*t*, 2H, $-CH_2-N_3$); 3.57–7.71 (*m*, 12H, $-CH_2-CH_2-O-$, $-CH_2-CH_2-N_3$ and $-O-CH_2-CH_2-S-$). ^{13}C NMR ($CDCl_3$) δ ppm: 29.0 ($-CO-CH_3$); 30.7 ($-CH_2-S-$); 50.8 ($-CH_2-N_3$); 69.9, 70.2, 70.5, 70.8, 70.9 ($-CH_2-CH_2-O-$ and $-CH_2-CH_2-N_3$); 195.6 (–CO–S–). IR (cm^{-1}): 2866, 2098, 1688, 1098, 624.

2.6. Synthesis of azide modified disulfide containing TEG ($(N_3$-TEG-S$)_2$) (5)

Sodium methoxide (0.86 g, 2 equiv., 15.9 mmol) was added to a solution of **4** (2.20 g, 7.93 mmol, 1 equiv.) in anhydrous methanol (50 mL). After stirring at room temperature for 2 h, DMSO (1.2 mL) was added to the reaction mixture for further stirring of 20 h. Amberlyst 15 was used to neutralize the reaction solution until it reached pH 7 and then filtered off. The solvent was removed under reduced pressure and the product **5** (1.734 g, 93%) was purified by column chromatography (MeOH/$CHCl_3$, 1:30 to 1:40). ^{1}H NMR ($CDCl_3$) δ ppm: 2.88 (*t*, 4H, $-CH_2-S-$); 3.38 (*t*, 4H, $-CH_2-N_3$); 3.60–3.69 (*m*, 20H, $-CH_2-CH_2-O-$ and–O–CH_2-CH_2-S-); 3.73 (*t*, 4H, $-CH_2-CH_2-N_3$). ^{13}C NMR ($CDCl_3$) δ ppm: 38.6 ($-CH_2-S-$); 50.8 ($-CH_2-N_3$); 69.8, 70.2, 70.5, 70.7, 70.8, 70.9 ($-CH_2-CH_2-O-$ and $-CH_2-CH_2-N_3$). IR (cm^{-1}): 2865, 2099, 1113. ESI- MS (m/z): calculated for $C_{16}H_{32}N_6O_6S_2$ $(MNa)^+$ 491.1723, found 491.1717.

2.7. Synthesis of 1, 2, 3-triazole cross-linked disulfide containing PEG–PEI-copolymer (6)

Propargyl bromide solution in 80% toluene (450 μL, 4.18 mmol, 10 equiv.) was added dropwise at 0 °C to a solution of poly(ethylene imine) (2 kDa) (0.853 g, 0.418 mmol, 1 equiv.) in methanol (5 mL) and stirred for 48 h. After that the azide modified disulfide containing TEG **5** (0.983 g, 4.18 mmol, 10 equiv.) in DMSO (1 mL) and also the sodium ascorbate and $CuSO_4$ aqueous solution were added dropwise to the reaction mixture and stirred further for 7 d. The copolymer was first purified by membrane dialysis against DI water (Mw Cutoff = 1000) and then centrifuged with tube vivaspin 6 (Mw Cutoff = 100,000). The filtrate was collected and lyophilized to obtain the product as a brown powder (765 mg, 38% yield). ^{1}H NMR (D_2O) δ ppm: 2.4–3.3 (*m*, $-CH_2-CH_2-N-$); 3.3–4.2 (*m*, $-CH_2-CH_2-O-$); 8.1 (*s*, H in triazole ring).

2.8. Polymer characterization

2.8.1. Nuclear magnetic resonance spectroscopy (NMR)

^{1}H NMR spectra of the polymers were recorded in D_2O (Merck, Darmstadt, Germany) on an Eclipse+ 500 spectrometer from JEOL (Tokyo, Japan) at 500 MHz. Spectra were evaluated with the NMR data processing program Delta Version 4.3.6.

2.8.2. Size exclusion chromatography (SEC)

The SEC setup consisted of a HPLC Pump L-6000 from Merck-Hitachi (Darmstadt, Germany) and a Merck-Hitachi autosampler AS-200A. Polymers were detected by a differential refractive index (RI) detector RI-71 from Merck. The SEC columns Suprema precolumn and SupremaMax 100 in combination with SupremaMax 3000 were from Polymer Standard Service (Mainz, Germany). The eluent, namely 1% formic acid solution, was prepared with pure water and degassed with an ultrasonic bath for 60 min per 2.5 L. A flow rate of 0.5 mL/min was applied. MWs were calculated with a relative method using RI-Detector and poly(ethylene imine)s with known molecular weights of 0.8, 2, 5 and 25 kDa were used as standards for calibration.

2.9. Polymer degradation studies

Degradation of the polymer was carried out in 5% glucose solution (pH 7.4) at room temperature in presence of 100 mM DTT. Size exclusion chromatography with the relative method as described above for polymer characterization with calibration of PEI-standards was performed for detection. Degradation solutions were measured at 0 h, 2 h, and 24 h.

2.10. Formation of polyplexes

The polyplexes consisting of plasmid DNA and polymers including either pure PEI (2k, 25k) or PEG–PEI-copolymer were prepared in a sterile isotonic glucose solution (5%) at pH 7.4 as described previously (Kleemann et al., 2004). Briefly, indicated amount of pDNA was added rapidly to the polymer solution and mixed by vigorous pipetting. After incubation for 20–30 min at room temperature the polyplexes were used. For the AFM measurement the DNA concentration in the complex solution was 1.5 μg/mL at a polymer nitrogen to DNA phosphate ratio (*N*/*P*) of 7. For other investigations the concentration of polymer solution was adjusted to the amount of DNA (20 μg/mL in polyplex solutions for *in vitro* experiments) to achieve *N*/*P* ratios from 0.5 to 10.

2.11. Gel retardation assay

Agarose gel electrophoresis was performed as previously described (Petersen et al., 2002). Briefly, polyplex solutions were incubated for 30 min at different *N*/*P* ratios. 25 μL aliquots were loaded onto a 1% agarose gel containing 60 μg ethidium bromide. Gels were run for 1 h at a voltage of 80 V in TBE buffer solution and then scanned with a Biometra gel analyzing system (BioDoc Analyzer, Biometra, Göttingen, Germany) for visualization. Polyplex solutions prepared with pure PEI (2k, 25k) were used as control.

2.12. Determination of polyplex size and zeta potential

The hydrodynamic diameters as well as the zeta potentials of freshly prepared polyplexes with herring testes DNA and polymers (PEI 2k, PEI 25k or PEG–PEI-copolymer) were measured in dynamic light scattering (DLS) using a Zetasizer Nano-ZS from Malvern Instruments (Herrenberg, Germany) equipped with a 4 mW He–Ne laser at a wavelength of 633 nm at 25 °C (Neu et al., 2006). Scattered light was detected at a 173° backward scattering angle with automatic measurement position and automatic laser attenuation. The viscosity and refractive index of pure water at 25 °C were used for data analysis. Hydrodynamic diameters were measured in micro cells with 50 μL sample solutions and zeta potential was measured in a folded capillary cell after 7:15 dilution with 5% glucose solution. Results were calculated using DTS software v4.10 and values are given as the mean of at least five measurements of 10 runs each. Measurements of polyplexes at each *N*/*P* ratio were repeated twice with a newly prepared sample.

2.13. Atomic force microscopy (AFM)

Images of the polyplexes were obtained using a JPK NanoWizard® AFM system (Germany), operating in tapping mode (Kleemann et al., 2004). All imaging was carried out at a scan speed of approximately 0.7 Hz with 512 × 512 pixel resolutions. Silicon nitride oxide-sharpened cantilevers were selected, operating at resonance frequencies of approximately 10 kHz. A sample volume of 40 μL was placed onto a 1 cm^2 disk of slide glass. 10 mM nickel chloride was added to the plasmid solution to facilitate immobilization of the plasmid on the glass surface. The freshly prepared polyplexes were imaged. To determine the mean particle size from

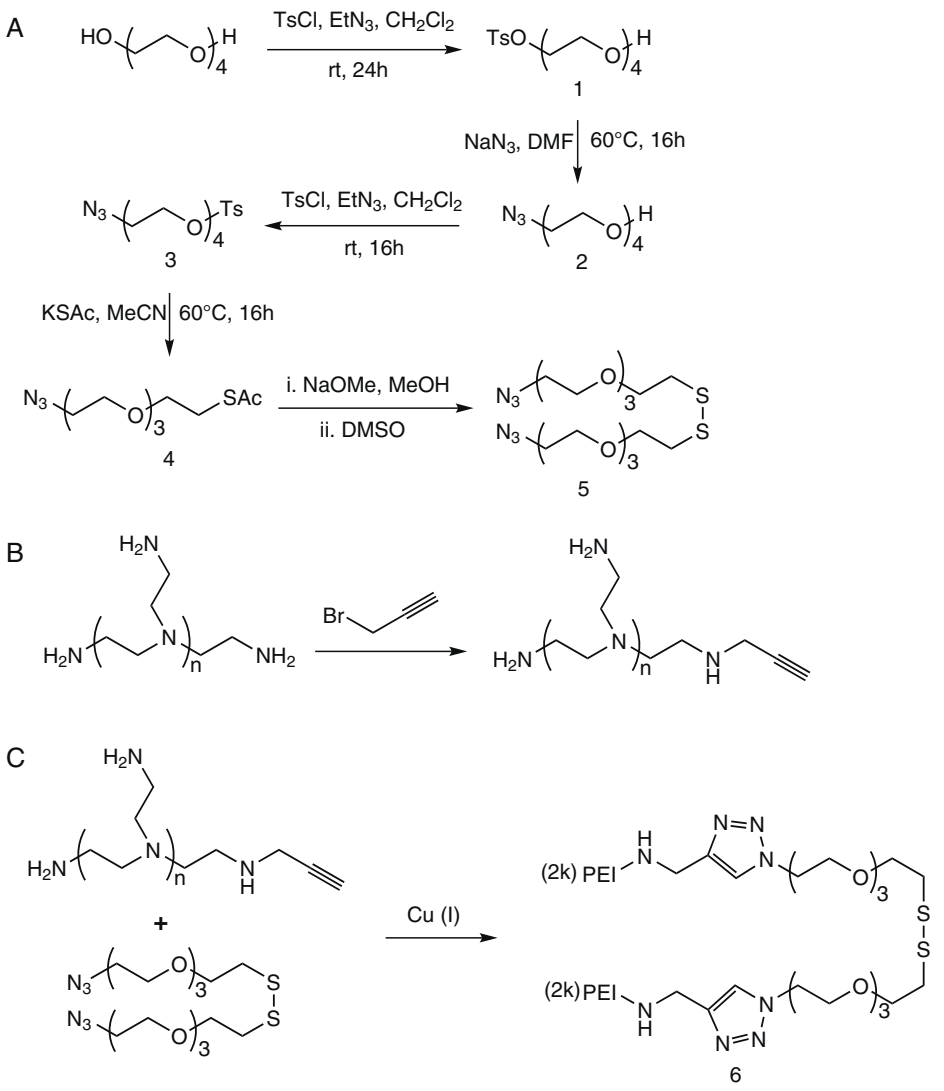

Fig. 1. Synthesis of the disulfide containing PEG–PEI copolymer cross-linked with click cluster: A. synthesis of azide modified disulfide containing PEG-linker; B. synthesis of alkyne functionalized PEI in a feed ratio of 1:10 between PEI and alkynes; C. copolymerization of PEI- and PEG-block using Huisgen 1, 3-dipolar cycloaddition (model structures are given in B and C).

these images, the diameters at about half-height of at least 30 polyplexes were analyzed.

2.14. Cell culture

2.14.1. Cytotoxicity determination

MTT assay was performed in L929 mouse fibroblasts as reported previously (Fischer et al., 2003). Briefly, L929 fibroblasts were seeded at a density of 8000 cells/well in 96-well plates (NUNC, Wiesbaden, Germany) and incubated for 24 h. Serial solutions of the copolymers (0.15–5000 μg/mL in cell medium) were added and incubated for 4 h. The medium was then replaced with 200 μL fresh medium and 20 μL/(3-(4,5-dimethylthiatol-2-yl)-2,5-diphenyl tetrazolium bromide)/well (MTT, Sigma, Seelze) and further incubated for 4 h. After removing the unreacted dye 200 μL DMSO were added. The ELISA reader Titertek Plus MS 212 (ICN, Eschwege, Germany) was used to measure the absorption at 570 nm and the background correction at 690 nm. The relative cell viability (%) was calculated by: absorption $_{test}$/absorption $_{control}$ × 100%. Wells containing cell culture medium without polymers were used as control. The values of the polymers were fitted to a logistical sigmoidal function using Origin® v 7.0 (OriginLab, Northampton, MS) and IC_{50} values were calculated as recently reported (Kunath et al., 2003) and given as mean ± SD (n = 4).

2.14.2. Transfection efficiency measurements

Cells were seeded in 96 well plates at a density of 6000 cells/well 24 h prior to experiments. Medium was aspirated and exchanged with 75 μl fresh medium. 25 μl of freshly prepared polyplexes were added to each well and incubated for 4 h. After replacement with 200 μl fresh medium, cells were further grown for 44 h. Transfection activity was measured according to the protocol provided by Promega (Madison, WI, USA). Relative light units were measured in a FLUOstar Optima (BMG labtech, Offenburg, Germany). Protein was determined using a BCA protein assay kit (Pierce Thermo Scientific, Rockford, IL, USA).

3. Results and discussion

3.1. Synthesis

Synthesis of the biodegradable PEG–PEI copolymer was achieved by coupling a disulfide containing short chain PEG (TEG) and a LMW branched PEI using a Huisgen 1,3-dipolar cycloaddition.

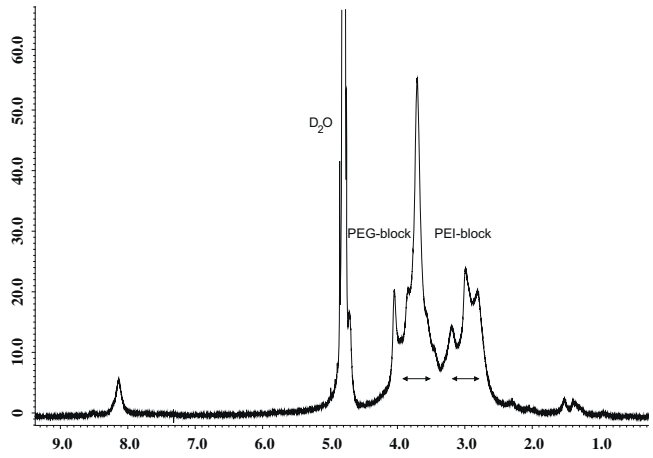

Fig. 2. ^{1}H NMR spectrum of the PEG–PEI copolymer in D_2O.

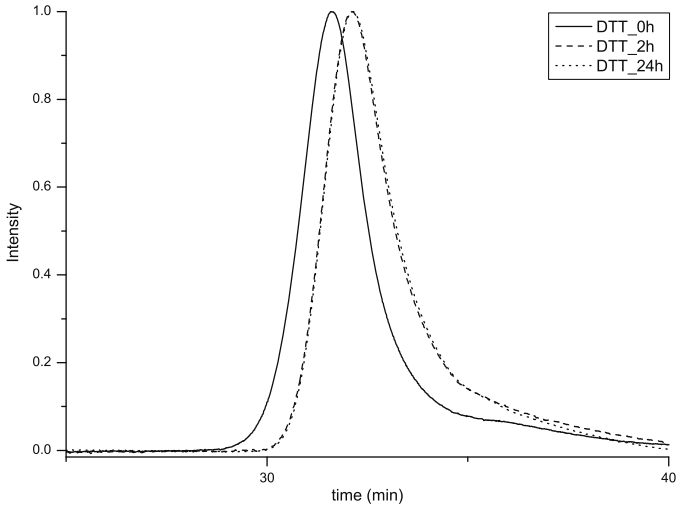

Fig. 3. Degradation behavior of PEG–PEI copolymer in presence of 100 mM DTT. The elution time correlates with the calculated molecular mass sequence by means of calibration with PEI-Standards.

The synthesis procedure consisted of three steps: PEG containing linker synthesis, synthesis of alkynes functionalized PEI and copolymerization of PEI- and PEG-block.

As shown in Fig. 1A, biodegradable disulfide bonds were first introduced into the PEG chain to generate the disulfide containing PEG-linker. One of the hydroxyl groups at the end of the TEG chain was modified with an azide group (Li et al., 2005) to create a stable triazol bond with alkynes between PEG and PEI. The other hydroxyl group was substituted with a thioacetate group (Li et al., 2005) and then reduced to thiol groups, which was allowed to be oxidized to disulfide in the air. The addition of DMSO enhanced the rate of oxidization (Tam et al., 1991). The obtained PEG-Linker was very clean after purification with column chromatography and characterized by NMR, FT-IR and ESI-MS. To couple with PEG-block, PEI 2k was modified with alkynes in a feed ratio of 1:10 (Fig. 1B). The excess amount of the alkyne groups in comparison to PEI 2k was used to increase the possible reaction conversion. The modification with feed ratios of 1:5 and 1:7 was also performed, but no significant increase of the molecular weight of the product was observed (data not shown). Therefore, an appropriate excess of the functional groups (hereby ≥10-fold) seemed to be required for the PEI-block for an efficient coupling with the PEG-block. Without further purification the synthesized disulfide containing PEG-linker was added into the reaction solution with the feed ratio of 1:1 between azide and alkyne (Fig. 1C). A catalytic amount of Cu (II) and sodium ascorbate was used to generate Cu (I) *in situ* to start the click reaction. An appropriate amount of water was added to dissolve sodium ascorbate.

The copolymer structure was studied by ^{1}H NMR (Fig. 2). Since there are some overlaps between signals of PEI-block and those of PEG-block, the peaks from 3.36 to 4.20 ppm and from 2.40 to 3.36 ppm are integrated as PEG-block and PEI-block, respectively. Their molar ratio via this estimation of their integrals amounts to, therefore, approximately 1:8.6. The synthesized copolymer was totally water soluble, which is suitable for drug delivery. The purification of the copolymer was performed by dialysis. GPC chromatogram indicated a pure copolymer with a molecular weight about 22 kDa.

It was reported that click chemistry used as linker chemistry has played a pivotal role in many areas of research, such as drug delivery and nanomedicine (Hein et al., 2008). In this report, click chemistry has successfully given a further proof of joining two homopolymer blocks together to form block copolymers, which was difficult to be realized by copolymerization with functional groups using other chemistry linkage. Irrespective of the linker chemistry, the copolymerization between PEG-linker and PEI-block was accomplished only in one step at room temperature without heating or removing water and oxygen, and was carried out efficiently, since about 86% PEG-linker were immobilized on the PEI-block according to the ^{1}H NMR-spectrum. The copper catalyst directed only the formation of the 1,4-regioisomer according to a previous report (Hein et al., 2008), which indicated that no complicated polymer structure resulting from cross-linking would occur.

3.2. Degradation with DTT

The degradation behavior of the synthesized PEG–PEI copolymer in 5% glucose was investigated by GPC (Fig. 3). Since the 1,2,3-triazole ring is very stable in typical biological conditions (Hein et al., 2008), such as reducing environment, the degradation behavior could only result from cleavage of disulfide bonds. In presence of dithiothreitol (DTT) with a final concentration of 100 mM the disulfide bonds were cleaved rapidly within 2 h. After 24 h degradation the GPC chromatogram was found almost overlapped with the one at the degradation time of 2 h, which indicated a total degradation of the PEG–PEI copolymer within a short period. In comparison to the molecular weight of the PEG–PEI copolymer before degradation (about 22 kDa), the degradation product after 24 h has a Mw of about 4 kDa, which is in correspondence with the calculation for the smallest PEG–PEI block of the copolymer with NMR-Spectrum. Since the structure of the degradation product contained not only PEI but also triazole coupled PEG, the degradation product has a higher molecular weight than PEI 2k. In summary, it was observed that the disulfide cross-linked PEG–PEI copolymer could be completely degraded within 2 h in presence of reducing reagent of DTT. In a reducing environment *in vivo* this rapid cleavage of disulfide bonds will ensure DNA release from complexes efficiently to facilitate the following nuclear import and gene expression. It can also reduce the cytotoxicity of PEG–PEI, since the low molecular weight degradation products can be more easily transported out of the cell.

3.3. DNA complexation

The condensation of DNA into small particles is a prerequisite for polycationic plasmid delivery. The formation of polyelectrolyte complexes between PEI and DNA is based on electrostatic interactions between the polymers' nitrogen atoms and the phosphate groups of the nucleotides. To evaluate the DNA condensation ability

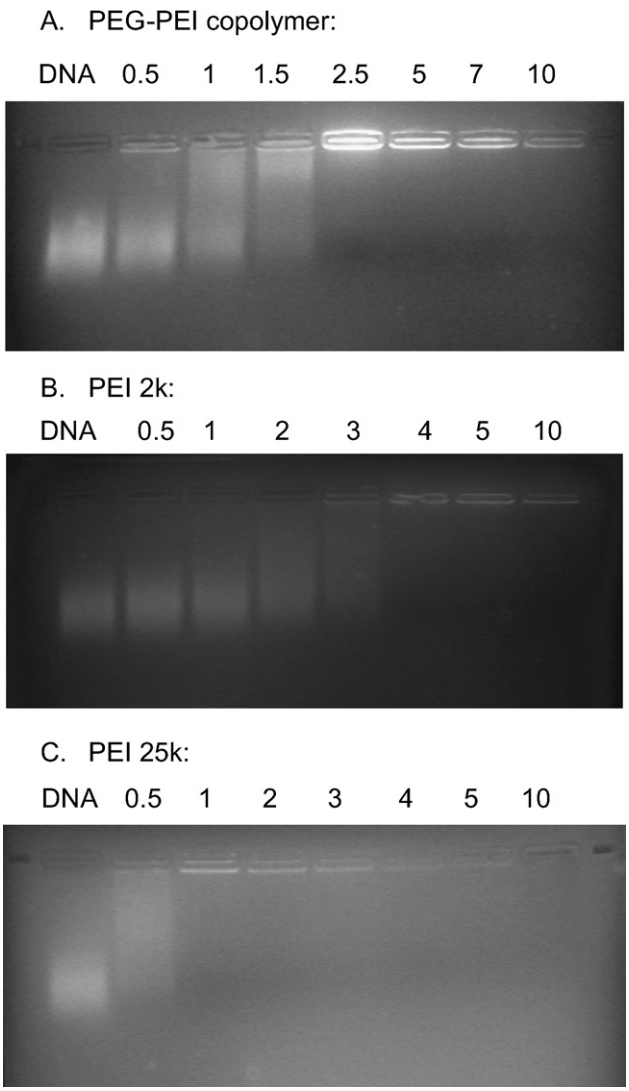

Fig. 4. Gel retardation assay of PEG–PEI polyplexes (A) and unmodified PEI polyplexes (B and C) formed with herring testes DNA.

of this PEG–PEI copolymer, its polyplexes in comparison of polyplexes from PEI 2k and PEI 25k were prepared at different *N*/*P* ratios and investigated by agarose gel electrophoreses. The PEI content in copolymer with the value of 34.5% was determined by copper assay (Harpe et al., 2000) in correlation with the concentration of PEI 25k standards using for calculation of the *N*/*P* ratios (data not shown).

From the results of gel retardation assay (shown in Fig. 4), the polyplexes prepared with copolymer have a obvious retardation at *N*/*P* 2.5, which indicated that the synthesized PEG–PEI copolymer could condense DNA around *N*/*P* 2.5. A total condensation appeared at about *N*/*P* 10, where the retarded polyplexes showed almost no fluorescence because no more DNA was intercalated by ethidium bromide. In comparison with the results from pure PEI-block, polyplexes prepared with PEI 2k showed a lower DNA condensation at about *N*/*P* 4. Polyplexes prepared with PEI 25k exhibited better condensation ability at about *N*/*P* 1. This could probably be explained by PEGylation. Due to the shielding effect of PEG-block the ability of the copolymer to condense DNA is slightly reduced compared with PEI 25k. PEI 2k showed the lowest ability to condense DNA because PEI 2k has the lowest Mw and therefore the shortest chains to cover DNA efficiently.

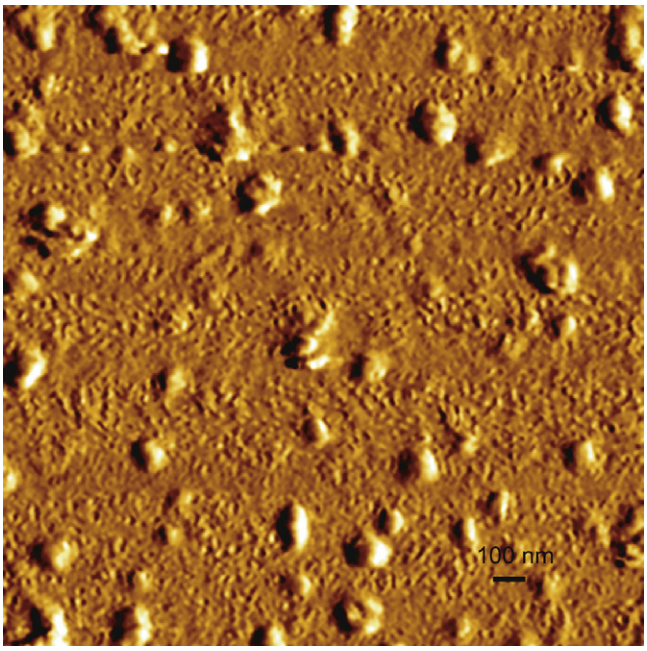

Fig. 5. AFM image of polymer/plasmid DNA complexes at *N*/*P* ratio 7 in 5% glucose at pH 7.4. Scale bars are equivalent to 100 nm. The color-encoded height scale extends 6 nm.

3.4. Size and morphology of the complexes

As reported in the literature, zeta potential and size of the polyplexes are important factors in facilitating gene transfer in cells (Fischer et al., 2000; Kircheis et al., 2001).

The size of the polyplexes prepared with PEG–PEI copolymer as well as with pure PEI (2k, 25k) is shown in Table 2. The polyplex sizes are all in the range from 80 to 130 nm in 5% glucose at pH 7.4. At lower *N*/*P* ratios like *N*/*P* 2.5 and 5 PEG–PEI copolymer showed smaller particle sizes than PEI 25k polyplexes, at higher *N*/*P* ratios PEI 25k polyplexes showed smaller sizes, indicating better DNA condensation. It is possible that at higher *N*/*P* ratios PEG component exhibited more efficiency of surface shielding and PEG spacer affected the condensation ability, resulting in enhanced polyplex sizes. In general, the disulfide-containing PEG–PEI copolymer was able to bind plasmid DNA efficiently to yield small polyplexes.

The surface charge of the polyplexes was investigated via zetapotentials using laser Doppler anemometry. It was reported that whilst naked DNA had a highly negative zeta potential of −33 mV (Kleemann et al., 2005), the polyplexes displayed positive zeta potentials due to an excess of the polycation. Table 1 shows the zetapotentials of PEG–PEI copolymer polyplexes and pure PEI polyplexes. PEG–PEI polyplexes showed positive zeta potentials in range of 10 to 30 mV(±3 mV) from *N*/*P* 2.5 to *N*/*P* 20, which is on average lower than the observed values of PEI 25k polyplexes from 30 to 50 mV (±1 mV). This could probably be caused by surface shielding of the PEG block. Therefore, it is assumed that PEG might partially appear at the particle surface, since PEI contributed to the positive surface charge for the polyplexes.

Atomic force microscopy was reported to be suitable for studying the morphology and size of polymer–DNA complexes (Kleemann et al., 2004). AFM imaging of PEG–PEI polyplexes formed with pDNA was carried out to support the size measurements by DLS and displayed the morphology of the polyplexes. AFM images of polyplexes resulting from PEG–PEI copolymer and pDNA at *N*/*P* 7 is shown in Fig. 5 with a scan size 2 μm × 2 μm. The pDNA was highly condensed with the PEG–PEI copolymer,

Table 1
Surface charge of PEG–PEI polyplexes and pure PEI 2k and PEI 25k polyplexes.

PEG–PEI	*N*/*P*	1	2.5	5	7	10	15	20
	Mean	-12.5 ± 5.7	19.1 ± 0.8	33.7 ± 1.3	24.8 ± 3.0	25.9 ± 0.6	22.7 ± 1.3	17.2 ± 3.2
PEI2k	*N*/*P*	2.5	7	10	15	20		
	Mean	14.7 ± 1.3	16.1 ± 5.2	30.1 ± 6.4	29.8 ± 6.2	32.9 ± 5.7		
PEI25k	*N*/*P*	0.5	1	2	4	7		
	Mean	-14.8 ± 1.4	-14.5 ± 1.3	31.1 ± 0.9	44.0 ± 1.0	49.9 ± 1.4		

Table 2
Hydrodynamic diameters of PEG–PEI polyplexes in 5% glucose solution compared with pure PEI 2k and PEI 25k polyplexes.

	N/*P*	2.5	5	7	10	15	20
PEG–PEI	Mean	93.5 ± 4.6	71.8 ± 4.4	125.1 ± 3.4	114.0 ± 1.0	124.6 ± 2.9	91.3 ± 2.2
	PDI	0.137 ± 0.048	0.241 ± 0.043	0.219 ± 0.016	0.182 ± 0.011	0.203 ± 0.020	0.169 ± 0.039
PEI2k	Mean	109.0 ± 12.1	74.4 ± 2.9	119.0 ± 4.0	95.4 ± 4.3	95.5 ± 1.6	184.5 ± 8.3
	PDI	0.407 ± 0.046	0.180 ± 0.026	0.260 ± 0.028	0.342 ± 0.096	0.238 ± 0.009	0.465 ± 0.030
PEI25k	Mean	130.7 ± 3.2	119.1 ± 27.3	78.3 ± 8.3	79.4 ± 9.4	107.2 ± 32.4	65.8 ± 15.2
	PDI	0.287 ± 0.019	0.272 ± 0.100	0.388 ± 0.080	0.258 ± 0.122	0.176 ± 0.033	0.389 ± 0.067

forming spherical particles with sizes of approximately 100 nm, since pDNA alone exhibited a relaxed, open loop structure with little twisting of the strands as shown in the previous report (Kleemann et al., 2004). Both results from DLS data and AFM images indicate a good condensation ability of the copolymer to DNA.

3.5. Cytotoxicity

The metabolic activity of polymer treated L929 cells was tested using a colorimetric MTT assay (Mosmann, 1983). The cytotoxicity of the PEG–PEI copolymer was compared with the toxicity of the pure PEI 2k and PEI 25k. As shown in Fig. 6, PEI 25k exhibited an IC_{50} value of about 10 μg/mL which is in correspondence with previous reports (Neu et al., 2007b), and PEI 2k exhibited an IC_{50} value of about 487 μg/mL. The comparison of the IC_{50} of PEG–PEI polymer with pure PEI showed that the PEG–PEI copolymer with IC_{50} value of 227 μg/mL is about 22-fold less toxic than PEI 25k and 2-fold more toxic than non-toxic PEI 2k. Hence, the modification of PEI with PEG may display sufficient shielding and the rapid degradation of disulfide bonds may also play a role for reducing the toxicity of the copolymer (Table 2).

3.6. Transfection efficiency

Transfection experiments were performed in L929 fibroblasts with plasmid DNA encoding for luciferase with different *N*/*P* ratios. As shown in Fig. 7, PEI 2k exhibited low transfection efficiency at the level of free DNA with only a slight increase at high *N*/*P* ratios. This result is according to the literature (Godbey et al., 1999b) and can be explained by a decreased protection of DNA by low molecular weight PEI. After coupling of small PEG chains and cross-linking by disulfide bridges an increased transfection efficiency at optimal *N*/*P* ratio can be obtained. The molecular weight of the PEG–PEI hereby is similar to that of PEI 25k. At optimal *N*/*P* ratio, PEG–PEI reaches approximately 17% of the transfection efficiency of PEI 25k. With increasing *N*/*P* ratio, cells are better transfected which can be explained by a higher stability of DNA in the polyplex and therefore a better protection of DNA. Due to the shielding effect of PEG, transfection efficiency of PEGylated PEIs are often reported to be lower than pure PEI of the same size, but also cytotoxicity is

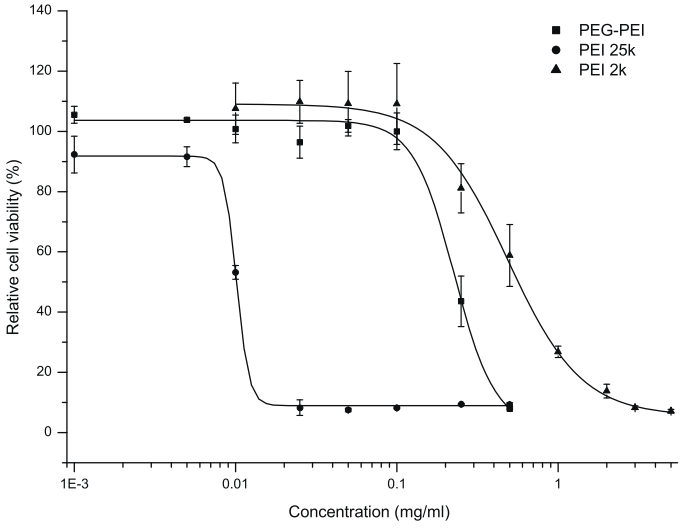

Fig. 6. Cytotoxicity of PEG–PEI copolymer in comparison to PEI 25k and PEI 2k as determined by MTT assay.

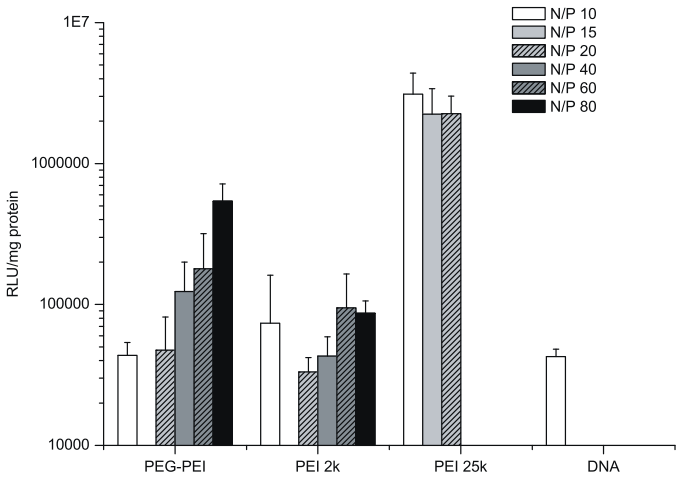

Fig. 7. Transfection efficiency of PEG–PEI copolymer in comparison to PEI 2k and PEI 25k in L929 fibroblasts with plasmid DNA encoding for luciferase with different *N*/*P* ratios from 10 to 80.

much lower and circulation times *in vivo* can be prolonged (Kichler, 2004). By using a low molecular weight PEI coupled with disulfide containing PEG, several advantages are achieved: (1) transfection efficiency of low molecular weight PEI could be enhanced, (2) PEG leads to a shielding effect of highly positive charges and therefore enhances stability and limits cytotoxic side effects, (3) low molecular weight PEI alone could be shown to be less toxic than high molecular weight PEI, and (4) disulfide bridges lead to degradability and with our polymer to low molecular weight PEI products which can be more easily transported out of the cell without accumulation.

4. Conclusions

A high molecular weight biodegradable disulfide containing PEG–PEI copolymer consisting of low molecular weight building blocks was synthesized and characterized with NMR and GPC. For block-copolymerization of PEG and PEI, click chemistry was used and offered easier synthetic routes, high cross-linking efficiency, milder reaction conditions, and an easier purification process, compared with other block copolymer synthesis. Additionally, this PEG–PEI copolymer showed a promising feature for effective gene delivery. A rapid and total degradation of the PEG–PEI copolymer within 2 h in the presence of reducing agent was observed. It indicated a rapid DNA releasing ability to facilitate the following gene expression and a reduced toxicity by disulfide bond cleavage into small PEI-products. Gel retardation assay demonstrated strong DNA condensation abilities of the PEG–PEI copolymer by forming nanosized polyplexes at low *N*/*P* ratios. The DLS and AFM measurements showed further proofs for the small size and morphology of the resulting polyplexes. MTT-assay indicated that the cytotoxicity of the synthesized PEG–PEI copolymer is comparable with non-toxic PEI 2k and much less toxic than PEI 25k. After coupling of small PEG chains and cross-linking by disulfide bridges, an increased transfection efficiency in comparison to PEI 2k and free plasmid DNA could be obtained and approximately 17% of the transfection efficiency of PEI 25k was reached.

In summary, this disulfide containing biodegradable PEG–PEI copolymer synthesized by click chemistry is a promising candidate for application in *in vivo* gene delivery. This method offers the possibility of coupling LMW PEGylated PEI into HMW PEGylated PEI containing totally biodegradable disulfide bonds. The structure of the polymer can still be modified by changing the PEG chain length or PEGylation degree. Further studies for improving the pharmaceutical properties such as transfection efficiency will be very interesting.

Acknowledgment

The author wants to thank Thomas Betz for the AFM measurements.

References

Dedola, S., Nepogodiev, S.A., Field, R.A., 2007. Recent applications of the Cu^{I}-catalysed Huisgen azide-alkyne 1,3-dipolar cycloaddition reaction in carbohydrate chemistry. Org. Biomol. Chem. 5, 1006–1017.

Fischer, D., Bieber, T., Li, Y., Elsässer, H., Kissel, T., 1999. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. Pharm. Res. 16, 1273–1279.

Fischer, D., Harpe, A.v., Kissel, T., 2000. Polyethylenimine: polymer structure influences the physicochemical and biological effects of plasmid/PEI complexes. In: Park, K.D., et al. (Eds.), Biomaterials and Drug Delivery toward New Millenium. , pp. S195–S211.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 24, 1121–1131.

Forrest, M.L., Koerber, J.T., Pack, D.W., 2003. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. Bioconjug. Chem. 14, 934–940.

Gao, C.X., Kim, K.-S., Liu, D., 2007. Nonviral gene delivery: what we know and what is next. AAPS J. 9, E92–E103.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999a. Poly(ethylenimine) and its role in gene delivery. J. Control. Release 60, 149–160.

Godbey, W.T., Wu, K.K., Mikos, A.G., 1999b. Size matters: molecular weight affects the efficiency of poly(ethylene imine) as a gene delivery vehicle. J. Biomed. Mater. Res. 45, 268–275.

Gosselin, M.A., Guo, W., Lee, R.J., 2001. Efficient gene transfer using reversibly crosslinked low molecular weight polyethylenimine. Bioconjug. Chem. 12, 989–994.

Harpe, A., Petersen, v., Li, H., Kissel, Y.T., 2000. Characterization of commercially available and synthesized polyethylenimines for gene delivery. J. Control. Release 69, 309–322.

Hein, C.D., Liu, X.-M., Wang, D., 2008. Click chemistry, a powerful tool for pharmaceutical sciences. Pharm. Res. 25, 2216–2230.

Jiang, X., Liu, J., Xu, L., Zhuo, R., 2011. Disulfide-containing hyperbranched polyethylenimine derivatives via click chemistry for nonviral gene delivery. Macromol. Chem. Phys. 212, 64–71.

Kichler, A., 2004. Gene transfer with modified polyethylenimines. J. Gene Med. 6, S3–S10.

Kichler, A., Chillon, M., Leborgne, C., 2002. Intranasal gene delivery with a polyethylenimine–PEG conjugate. J. Control. Release 81, 379–388.

Kircheis, R., Blessing, T., Brunner, S., Wightman, L., Wagner, E., 2001. Tumor targeting with surface-shielded ligand—polycation DNA complexes. J. Control. Release 72, 165–170.

Kleemann, E., Dailey, L.A., Abdelhady, H.G., Gessler, T., Schmehl, T., Roberts, C.J., Davies, M.C., Seeger, W., Kissel, T., 2004. Modified polyethylenimines as nonviral gene delivery systems for aerosol gene therapy: investigations of the complex structure and stability during air-jet and ultrasonic nebulization. J. Control. Release 100, 437–450.

Kleemann, E., Neu, M., Jekel, N., Fink, L., Schmehl, T., Gessler, T., Seeger, W., Kissel, T., 2005. Nanocarriers for DNA delivery to the lung based upon a TAT-derived peptide covalently coupled to PEG–PEI. J. Control. Release 109, 299–316.

Kunath, K., Harpe, A., Fischer, v., Petersen, D., Bickel, H., Voigt, U., Kissel, K.T., 2003. Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine. J. Control. Release 89, 113–125.

Kursa, M., Walker, G.F., Roessler, V., Ogris, M., Roedl, W., Kircheis, R., Wagner, E., 2003. Novel shielded transferrin–polyethylene glycol–polyethylenimine/DNA complexes for systemic tumor-targeted gene transfer. Bioconjug. Chem. 14, 222–231.

Lee, Y., Koo, H., Jin, G.-W., Mo, H., Cho, M.Y., Park, J.-Y., Choi, J.S., Park, J.S., 2005. Poly(ethylene oxide sulfide): new poly(ethylene glycol) derivatives degradable in reductive conditions. Biomacromolecules 6, 24–26.

Lee, Y., Mo, H., Koo, H., Park, J.-Y., Cho, M.Y., Jin, G.-W., Park, J.-S., 2007. Visualization of the degradation of a disulfide polymer linear poly(ethylenimine sulfide), for gene delivery. Bioconjug. Chem. 18, 13–18.

Li, J., Crasto, C.F., Weinberg, J.S., Amiji, M., Shenoy, D., Sridhar, S., Bubley, G.J., Jones, G.B., 2005. An approach to heterobifunctional poly(ethyleneglycol) bioconjugates. Bioorg. Med. Chem. Lett. 15, 5558–5561.

Liu, J., Jiang, X., Xu, L., Wang, X., Hennink, W.E., Zhuo, R., 2010. Novel reduction-responsive cross-linked polyethylenimine derivatives by click chemistry for nonviral gene delivery. Bioconjug. Chem. 21, 1827–1835.

Lungwitz, U., Breunig, M., Blunk, T., Göpferich, A., 2005. Polyethylenimine-based non-viral gene delivery systems. Eur. J. Pharm. Biopharm. 60, 247–266.

Luten, J., van Nostrum, C.F., Smedt, S.C.D., Hennink, W.E., 2008. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. J. Control. Release 126, 97–110.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Neu, M., Fischer, D., Kissel, T., 2005. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J. Gene Med. 7, 992–1009.

Neu, M., Sitterberg, J., Bakowsky, U., Kissel, T., 2006. Stabilized nanocarriers for plasmids based upon cross-linked poly(ethylene imine). Biomacromolecules 7, 3428–3438.

Neu, M., Germershaus, O., Behe, M., Kissel, T., 2007a. Bioreversibly crosslinked polyplexes of PEI and high molecular weight PEG show extended circulation times *in vivo*. J. Control. Release 124, 69–80.

Neu, M., Germershaus, O., Mao, S., Voigt, K.H., Behe, M., Kissel, T., 2007b. Crosslinked nanocarriers based upon poly(ethylene imine) for systemic plasmid delivery: *in vitro* characterization and *in vivo* studies in mice. J. Control. Release 118, 370–380.

Ogris, M., Brunner, S., Schuller, S., 1999. PEGylated DNA/transferring-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 6, 595–605.

Peng, Q., Hu, C., Cheng, J., Zhong, Z., Zhuo, R., 2009. Influence of disulfide density and molecular weight on disulfide cross-linked polyethylenimine as gene vectors. Bioconjug. Chem. 20, 340–346.

Petersen, H., Fechner, P.M., Martin, A.L., Kunath, K., Stolnik, S., Roberts, C.J., Fischer, D., Davies, M.C., Kissel, T., 2002. Polyethylenimine-graft-poly(ethylene glycol) copolymers: influence of copolymer block structure on DNA complexation and biological activities as gene delivery system. Bioconjug. Chem. 13, 845–854.

Tam, J.P., Wu, C.-R., Liu, W., Zhang, J.-W., 1991. Disulfide bond formation in peptides by dimethyl sulfoxide scope and applications. J. Am. Chem. Soc. 113, 6657–6662.

Tang, G.P., Zeng, J.M., Gao, S.J., 2003. Polyethylene glycol modified polyethylenimine for improved CNS gene transfer: effects of PEGylation extent. Biomaterials 24, 2351–2362.

Tron, G.C., Pirali, T., Billington, R.A., Canonico, P.L., Sorba, G., Genazzani, A.A., 2008. Click chemistry reactions in medicinal chemistry: applications of the 1,3-dipolar cycloaddition between azides and alkynes. Med. Res. Rev. 28, 278–308.