Carbohydrate Polymers 79 (2010) 277–283

Contents lists available at ScienceDirect

Carbohydrate Polymers

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

21-Arm star polymers with different cationic groups based on cyclodextrin core for DNA delivery

Jianshu Li [a], Zhizhang Guo [a], Jianyu Xin [a], Guanglei Zhao [b], Huining Xiao [c,*]

[a] College of Polymer Science and Engineering, Sichuan University, Chengdu 610065, China
[b] College of Light Ind. and Food Sci., South China University of Technology, Guangzhou 510640, China
[c] Department of Chemical Engineering, University of New Brunswick, P.O. Box 4400, Fredericton, Canada NB E3B 5A3

ARTICLE INFO

Article history:
Received 1 July 2009
Received in revised form 24 July 2009
Accepted 3 August 2009
Available online 9 August 2009

Keywords:
β-Cyclodextrin
Star polymer
Gene delivery system
Cell transfection
Cytotoxicity

ABSTRACT

A series of cationic star polymers with 21 arms (21ACSPs) were synthesized through atom transfer radical polymerization using a β-cyclodextrin initiator with 21 initiation sites. Monomers containing primary, tertiary amino and quaternary ammonium groups were polymerized using 21Br-β-CD, Cu(I)Br and 2,2′-dipyridyl as initiator, catalyst and ligand, respectively. It was found that a co-solvent of 1-methyl-2-pyrrolidione and water (1:1) could facilitate the reaction, resulting in a well-controlled living polymerization. The conversion was up to 95% compared to the previously reported method (60%). AFM and DLS measurement revealed that 21ACSPs have the ability to condense the plasmid DNA (pCMV-Luc) to 80–180 nm. 21ACSPs with primary and tertiary amino groups exhibited higher cell transfection efficiency to CHSE-214 cells than 21ACSPs with quaternary ammonium groups. Meanwhile, all of the reported star polymers have no obvious cytotoxicity. The findings from this work are expected to be helpful for the development of efficient DNA delivery system.

1. Introduction

It has been realized that the lack of non-toxic and efficacious delivery systems is the greatest hurdle in exploiting the full potential of gene therapy. Over the past decade, nonviral DNA vectors have been demonstrated to be a safety-reliable substitute of viral vector for use in gene therapy (Li & Loh, 2008; Ooya et al., 2006; Opanasopit et al., 2009; Pack, Hoffman, Pun, & Stayton, 2005; Shen, Li, Tu, & Zhu, 2009). In addition to cationic lipids, cationic polymer is one of the typical kinds of nonviral DNA vector due to its capability to condense DNA into small particles termed polyplexes through electrostatic self-assembly (Merdan, Kopecek, & Kissel, 2002). The polyplex can bind to cell surfaces and be internalized into the cell through endocytosis. However, both extracellular and intracellular biological barriers can inhibit the approach of the therapeutic gene into the cell nucleus. Currently, the identified biological barriers include the systemic barriers (e.g., the gene targeting (Bellocq, Pun, Jensen, & Davis, 2003)) and the cellular barriers (e.g., the endosomal escape after endocytosis (Kichler, Leborgne, Coeytaux, & Danos, 2001) and the nuclear barrier (Bremner, Seymour, Logan, & Read, 2004)).

Synthetic cationic polymers can be tailored for various applications by choosing appropriate molecular weight (Yang, Li, Goh, & Li, 2007), suitable charge density (Reineke & Davis, 2003), coupling of cell or tissue specific targeting moieties, and performing other modification that afford them specific physicochemical or physiological properties (Merdan et al., 2002). Polyethylenimines (PEI), both branched and linear types, provide efficient transfection due to the well known "proton sponge property" (Boussif, Zanta, & Behr, 1996). However, it was found that the cytotoxicity of PEI is rather high, which may limit its further applications (Fischer, Bieber, Li, Elsasser, & Kissel, 1999). Poly(L-lysine)-based polymeric vectors are also widely investigated (Wolfert et al., 1999) due to their biodegradable property contributed by the peptide structure. Nevertheless, the DNA transfection efficiency of poly(L-lysine) based polymers is poor unless conjugating with specific ligands, such as transferrin or folate (Cho, Kim, Jeong, & Park, 2005), or with the help of endosomolytic or lysosomotropic agents such as chloroquine (Pouton et al., 1998). Dendrimers, which are spherical and hyperbranched, are also the effective cationic polymers for gene delivery. It was believed that the star shape of dendrimers determined their excellent transfection performance: DNA interact with the surface amino groups only, leaving the internal amino groups available for the neutralization of the acid pH within the endosomal or lysosomal compartment (Lee, Wang, & Low, 1996). It was also found the fractured dendrimers exhibit much higher levels (>50-fold) of reporter gene expression than that of intact dendrimers due to the better flexibility to complex DNA (Tang, Redemann, & Szoka, 1996). However, from a molecular structural

* Corresponding author. Tel.: +1 506 453 3532; fax: +1 506 453 3591.
E-mail address: hxiao@unb.ca (H. Xiao).

0144-8617/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2009.08.006

design point of view, the structure of dendrimers is rather congested; and the generation of the dendrimers is thus limited to form a star with more cationic groups. Also, the flexibility of dendrimers will decrease with the increase of their generations, whereas the star polymer have better flexibility due to the lack of the connection between each generation as that of dendrimers. This literally raises the demand on the synthesis of star polymers with a star shape similar to dendrimers but does not have a limitation of the length of its branches. Georgiou et al. (Georgiou, Phylactou, & Patrickios, 2006; Georgiou, Vamvakaki, Patrickios, Yamasaki, & Phylactou, 2004) did the first study about star polymers based on 2-(dimethylamino)ethyl methacrylate (DMAEMA) as gene vectors and compared them to a commercially available dendrimer (SuperFect). The overall transfection efficiency of the optimum star polymer to human cervical HeLa cancer cells was comparable to the SuperFect. A recent work reported by Yang et al., have revealed that a kind of cationic star polymer consisting of α-cyclodextrin core and oligoethylenimine arms could reduce the cytotoxicity but retain or even enhance the gene transfection efficiency of PEI(25 K) (Yang et al., 2007). Xu et al. (2009) also prepared star-shaped copolymer consisting of β-CD core and DMAEMA arms, with poly(poly(ethylene glycol)ethyl ether methacrylate)) P(PEGEEMA) as end blocks. The unique star-shaped architecture can reduce cytotoxicity and enhance the gene transfection efficiently compared with that of PEI (25 K). Thus further research on star polymer is expected to be promising as an effective DNA vector due to the various reasons addressed above.

Living polymerization, including atom transfer radical polymerization (ATRP), has been often used for synthesizing star polymers with well-defined structure, arm chain length in particular (Simms & Spinelli, 1997). In our previous study, a special ATRP initiator with 21 initiation sites (21Br-β-CD) was synthesized (Li & Xiao, 2005), and followed by the success in synthesizing a range of 21-arm cationic star polymer (Star-p(MeDMA)) with well-defined structures using the initiator (Li, Xiao, Kim, & Lowe, 2005). The polymerization was carried out in aqueous solution and the resulting star polymers are suitable for potential biomedical applications.

The Star-p(MeDMA) was synthesized from monomer with quaternary ammonium groups, which will not have the "proton sponge property" (Boussif et al., 1995) at physiological pH. Also, the moderate water solubility of the 21Br-β-CD was proved to be a limitation to obtain 21 arms star polymers (Li et al., 2005). In this work, we utilized a mixed solvent of 1-methyl-2-pyrrolidione and water to facilitate the living polymerization of a series of novel cationic star polymers with 21 arms (21ACSPs) through ATRP using 21Br-β-CD initiator. In addition, monomers bearing primary, tertiary amino and quaternary ammonium groups, which have been polymerized into linear polymers as DNA vectors and with suitable pK_a values (Rémy-Kristensen, Clamme, Vuilleumier, Kuhry, & Mély, 2001), were all used in an attempt to identify the polymer structure–bioactivity relation in terms of physicochemical properties, cell transfection efficiency and cytotoxicity.

2. Experimental

2.1. Materials

Heptakis[2,3,6-tri-*O*-(2-bromo-2-methylpropionyl)]-β-cyclodextrin(21Br-β-CD) was synthesized using an improved synthetic-route developed in our previous work. Ethylenediamine, glycidyl methacrylate, 2-(Dimethylamino)ethyl methacrylate (DMAEMA), *N*-[3-(Dimethylamino)propyl] methacrylamide (DMAPMAm) and [2-(methacryloyloxy)ethyl] trimethyl ammonium chloride (MeDMA) were obtained from Aldrich. Cu(I)Br, 2,2′-bipyridine (bpy), 1-methyl-2-pyrrolidione (NMP), branched polyethylenimine (PEI, 25 KDa), chloroquine and other regents were purchased from Aldrich and used without further purification. GPC standard samples, Pullulan (P-82, a linear macromolecular polysaccharide that consists of links of maltotriose) from P-5 to P-800, were obtained from Shodex. Spectra/Por dialysis membrane (MWCO 1000) was purchased from Spectrum Laboratories Inc. Distilled–deionized water was used in all the experiments.

Plasmid pCMV-Luc containing the luciferase as a reporter gene was obtained from Plasmid Factory, Bielefeld, Germany. CHSE-214 (Chinook salmon embryo) cell line was purchased from American Type Culture Collection (ATCC). Both luciferase assay kit and protein BCA assay kit were purchased from Sigma.

2.2. Synthesis and characterization of 21 arms cationic star polymers (21ACSPs)

First, the cationic monomer with primary amino group, i.e., [2-hydroxy-3-(2-aminoethyl) amino]propyl methacrylate (HAEAPMA), was synthesized as follows: Dropwise add 14.66 g glycidyl methacrylate into the mixture of 6.02 g ethylenediamine in 30 ml H_2O. The reaction was then carried out at room temperature for additional 45 min. The obtained HAEAPMA was precipitated and washed by cold diethyl ether (3 × 50 ml) (Anal. $C_9H_{18}N_2O_3$, C, H, N; FTIR (zinc selenide): 1717 cm^{-1} ($\nu_{C=O}$), 1042 cm^{-1}(ν_{C-N}); ^{1}H NMR (D_2O, 300 MHz): δ 2.72(N–H), δ 1.3(C–H).). The polymerizations of HAEAPMA as well as other cationic monomers with tertiary amino or quaternary ammonium groups, i.e., 2-(Dimethylamino) ethyl methacrylate (DMAEMA), *N*-[3-(Dimethylamino)propyl] methacrylamide (DMAPMAm) and [2-(methacryloyloxy)ethyl] trimethyl ammonium chloride (MeDMA), were conducted under the same conditions as those addressed in our previous paper (Li et al., 2005). The only difference was that the polymerization proceeded in a co-solvent of 1-methyl-2-pyrrolidione (NMP) and water at a ratio of 1:1 instead of water alone. The resulting 21ACSPs were purified with dialysis membrane (MWCO 1000) against water for 24 h prior to use.

^{1}H NMR spectra of the samples in D_2O were acquired using a Varian Unity 400 spectrometer operated at 300 MHz in order to quantify the monomer conversion in ATRP. The conversion of monomer was determined by comparing the peak integrals of the monomer vinyl signals at δ 5.5 and δ 6.0 to those of the methacrylate backbone at δ 0.5–1.1 and δ 1.5–2.0 (Li, Armes, Jin, & Zhu, 2003). Apparent charge density of the 21ACSPs was determined via colloidal titration using a Particle Charge Detector MÜtek PCD 03 (Herrsching, Germany). 0.2 ml of 0.1% polymer solution was added into the sample cell containing 9.8 ml water. The solution was titrated with a standard anionic polyelectrolyte (potassium polyvinyl sulphate (PVSK) solution) (concentration = 1 mM). The charge densities of the star polymers were estimated from the volume of the anionic polymer solution required to reach the end point during titration. Three repeats were conducted to acquire an average value for each sample. Gel permeation chromatography (GPC) (Pump: Waters 600E System Controller; Detector: Waters 410 Differential Refractometer) was carried out to determine their molecular weights with Ultrahydrogel 250 and Ultrahydrogel 500 columns at 40 °C and the flow rate at 0.7 ml/min. Water was used as an eluent. Calibration was made using standard Pullulan samples from P-5 to P-800. The standard sample aqueous solutions (0.2% w/v) were filtered with a 0.45 μm Nylon Cameo filter-syringe before the test.

2.3. Preparation of 21ACSPs/DNA polyplexes

Stock 21ACSPs and luciferase plasmid (pCMV-Luc) solutions were prepared in PBS buffer at pH = 7.4 at various molar ratios of N/P up to 10 prior to each experiment. Polyplex formation always utilized the solutions of equal volumes with the polymer solution

being added to the DNA solution, not the opposite sequence (Boussif et al., 1996). The mixed solution was vortexed and incubated at room temperature for 30 min before use. Polyplexes were freshly prepared before each individual measurement.

2.4. Zeta-potential measurements of 21ACSPs/DNA polyplexes

The DNA concentration was held constant at 20 μg/ml, whereas the N/P ratios of the 21ACSPs/pCMV-Luc polyplexes were varied. An electrophoresis meter DELSA 440SX (Coulter, USA) was used to determine the zeta-potential of the polyplexes in the presence of 0.1 mM NaCl in aqueous solution at 25 °C. The system was routinely calibrated using a −55 mV standard. Three repeats were conducted to acquire an average value for each test.

2.5. Particle characterization by atomic force microscopy (AFM) and dynamic light scattering (DLS)

Imaging samples of 21ACSP alone were prepared at a concentration of 100 μg/ml. Samples containing polyplexes in water were fixed at a final DNA concentration of 10 μg/ml. Samples were deposited onto the center of a freshly split untreated mica disk (Agar Scientific Inc.). Following the adsorption for 1–2 min at room temperature, excess fluid was taken off by absorption onto filter paper. The mica surface was dried at room temperature before imaging. A Nanoscope IIIa controller with a Multimode Pico-Force AFM (Veeco Instruments Inc., Santa Barbara, CA) was used. AFM imaging was performed in air in Multimode using silicon tapping tips (NP-S20, Veeco Instruments).

Dynamic light scattering (DLS) size determination: 21ACSP/DNA polyplexes were prepared as previous procedure and then diluted with PBS to a final concentration of 1 μg DNA/ml. Polyplex size determination was performed on a DynaPro-MS800 (Protein Solutions, Lakewood, NJ) with Dynamics v6.3 software. Samples were run in triplicate.

2.6. Transfection in vitro and cell viability

CHSE-214 cells were seeded in 12 well plates at a density of 50,000 cells per well one day before transfection. At the day of transfection, the cells have grown to approximately 60–80% confluence. Transfection experiments were performed with 4 μg of pCMV-Luc per well as follows: prepare polyplexes with adding appropriate amount of 21ACSP in 300 μl PBS buffer to 4 μg plasmid in 300 μl PBS buffer (pH = 7.4). Remove the medium and rinse the cells with 4 °C PBS (3 × 5 min × 2 ml). Add the polyplexes in 1 ml of fresh medium without FBS. Then the cells were incubated at room temperature for 4 h. After that, the medium was exchanged and the cells were incubated for additional 40 h. Luciferase gene expression was quantified using a Luciferase Assay Kit (Sigma) with a POLARstar OPTIMA luminometer (BMG LABTECH, USA). Results in relative light units per second (RLU/s) were converted into nanograms of luciferase by creating a calibration curve with luciferase standard (Sigma). Protein concentration in each sample indicating cell viability was determined using BCA Protein Assay Kit. The final transfection results were expressed in nanograms of luciferase per milligram of protein. pCMV-Luc alone was used as a control and data is reported from six separate experiments.

3. Results and discussion

3.1. Synthesis of 21ACSPs by ATRP in co-solvent

Living polymerization, including atom transfer radical polymerization (ATRP), has often been used for synthesizing star polymers with well-defined structure by both core-first and arm-first methods (Simms & Spinelli, 1997). In our previous work (Li & Xiao, 2005), we developed a novel ATRP initiator (21Br-β-CD) with 21 initiation sites based on β-cyclodextrin (β-CD). The 21 sites in β-CD are due to its cyclic oligosaccharide consisting of seven glucose units linked by α-1,4-glucosidic bonds and each unit having three substitutable hydroxyl groups. After preparing the initiator, we synthesized a cationic star polymer (Star-p(MeDMA)) in aqueous medium and obtained some positive results (Li et al., 2005). However, we found that the limited water solubility of 21Br-β-CD restricted its induction of ATPR in preparing the star polymers with a wide range of molecular weights. For example, at a higher initiator concentration of 100/1/2/5 ($[M]_0/[I]_0/[Cu(I)_0/[L]_0$) at 40 or 60 °C which was aimed at acquiring a star polymer with low molecular weight, the ATRP process, however, lost control and resulted in the polymer with very high molecular weight. The reason is simple: the 21Br-β-CD initiator could not be completely dissolved at this condition and the low initiator efficiency would generate much higher molecular weight with broad polydispersity. A lower initiator concentration at 500/1/2/5($[M]_0/[I]_0/[Cu(I)_0/[L]_0$) and a higher reaction temperature at 90 °C have been proved feasible to synthesize a star polymer with high molecular weight, e.g., 79,400, in our previous work.

However, star polymers with lower molecular weight (thus smaller star size) could facilitate the formation of DNA/polymer polyplexes with smaller size, which has been believed to be more desirable to achieve a high transfection efficiency (Kunath, von Harpe, Fischer, & Kissel, 2003);. Thus, we proposed to optimize the polymerization conditions based on 21Br-β-CD ATRP initiator by utilizing 1-methyl-2-pyrrolidione (NMP) as solvent. Preliminary experiment was performed in NMP alone, which led to a successful polymerization in homogeneous system and resulted in a star polymer with well-controlled structure. Having considered the solubility of hydrophilic cationic monomers, we tried NMP/water cosolvent and gradually reduced the NMP content in the entire system until the lowest ratio of NMP to water (1:1) was reached. Fig. 1 shows the chemical structures of these 21 arms cationic star polymers (21ACSPs) synthesized in the mixed solvent using various cationic monomers. As can be seen, all the 21ACSPs radiate from a common β-cyclodextrin core but have different types of amino functional groups, i.e., primary amino, tertiary amino and quaternary ammonium groups. It is also worth noting that the performance of Star-p(HAEAPMA) might be contributed from the secondary amino groups although they may not be as important as the primary amino ones due to the steric hindrance.

In this work, we fixed the ratio of monomer: initiator: catalyst: ligand at 100/1/2/5 for all the 21ACSPs except for Star-p(MeDMA) which was still synthesized at various ratios. Polymerization temperature was kept at 60 °C for all. An obvious change observed was

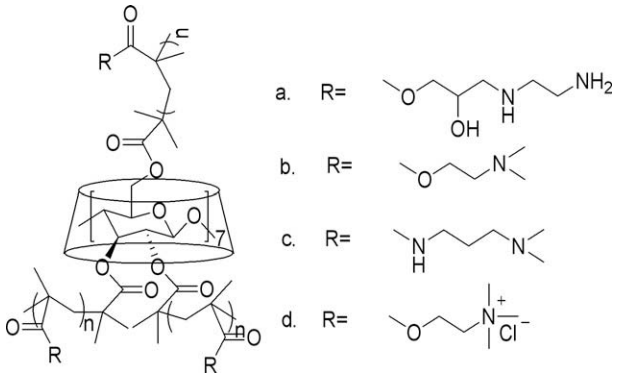

Fig. 1. Chemical structures of 21ACSPs. (a) Star-p(HAEAPMA); (b) Star-p(DMAEMA); (c) Star-p(DMAPMAm); (d) Star-p(MeDMA).

that the reaction system became homogenous rather than heterogeneous as previous systems due to the incomplete dissolvability of the initiator in water at 60 °C.

Table 1 lists the results of the polymerizations. As it can be seen, the homogenous polymerization system brought by NMP/water co-solvent resulted in two significant improvements compared with our previous report (Li et al., 2005): First, the molecular weights of the polymers with well-defined structure are close to the designed values at wide temperature and monomer concentration ranges. The previous reaction system in water can only obtain the well-defined star polymer at a high monomer concentration and a high temperature, i.e., 500/1/2/5 ($[M]_0/[I]_0/[Cu(I)_0/[L]_0$) at 90 °C, in which all the initiator can be totally dissolved at the beginning of the polymerization. Otherwise at low monomer concentration (thus high initiator concentration) and low temperature, the incomplete dissolved initiator would cause low initiation efficiency and result in unexpected high molecular weight (e.g., polymerization of MeDMA at 100/1/2/5 and 60 °C lead to a star polymer with molecular weight as high as 500,500 instead of the theoretical value of 15,600) (Li et al., 2005). As shown in Table 1, we synthesized MeDMA in the 1:1 NMP/water co-solvent at different monomer: initiator ratios, ranging from 20/1 to 250/1; and produced all Star-p(MeDMA)s with the molecular weights close to the designed values. Secondly, the monomer conversion is improved remarkably. The conversion of MeDMA polymerization in water alone varied from 39% to 70% under different conditions in our previous study (Li et al., 2005). The current co-solvent polymerization system enabled us to obtain star polymers with high monomer conversion up to 95%, which is comparable to the high conversion reported for MeDMA linear polymer (Li et al., 2003). The monomer conversion in the synthesis of star polymers is strongly dependent on the steric hindrance. Low initiation efficiency could lead to undesirable high molecular weight and congest star branches, thus preventing the monomers from approaching to initiation site and reducing the conversion. The polymerization in co-solvent eliminated the uncontrolled growth of the star branches, and thus increasing the accessibility of monomers toward initiation sites. It should also be noted that an appropriate polymerization time (e.g., 6 h) could decrease the coupling termination between two or more star polymers, which may generate a multiple-core star polymer as addressed elsewhere (Li et al., 2003; Ohno, Wong, & Haddleton, 2001).

Meanwhile, we found the charge densities of the 21ACSP were influenced significantly by their functional groups as shown in Table 1. The star polymers with primary amino groups and tertiary amino groups, i.e., Star-p(HAEAPMA), Star-p(DMAEMA) and Star-p(DMAPMAm), have much lower charge density than that of Star-p(MeDMA) with quaternary ammonium groups. The difference originates from their different principles in rendering the polymers cationic. The quaternary ammonium group carries a permanent cationic $-N^+(CH_3)_3Cl^-$ on its structure, whereas only part of the primary and tertiary amino groups in the star polymer could be positively charged by protonation process. It is to be noted here that direct titration method was used instead of reverse titration method to measure the surface charges of star polymers since the information is more important to evaluate their abilities to condense DNA.

Figs. 2 and 3 present the kinetic data of Star-p$(MeDMA)_3$ at an initial ratio of 250/1/2/5($[M]_0/[I]_0/[Cu(I)_0/[L]_0$) synthesized at 60 °C. A good linear relationship of the semilogarithmic plot of monomer concentration vs. time was observed, which implies that the radical concentration was constant throughout the polymeri-

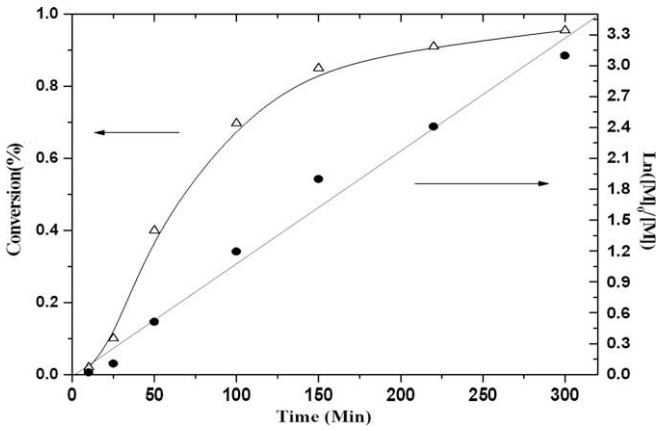

Fig. 2. Conversion vs. time data and semilogarithmic plot of monomer concentration vs. time for Star-p$(MeDMA)_3$ synthesized at 250/1/2/5 and 60 °C.

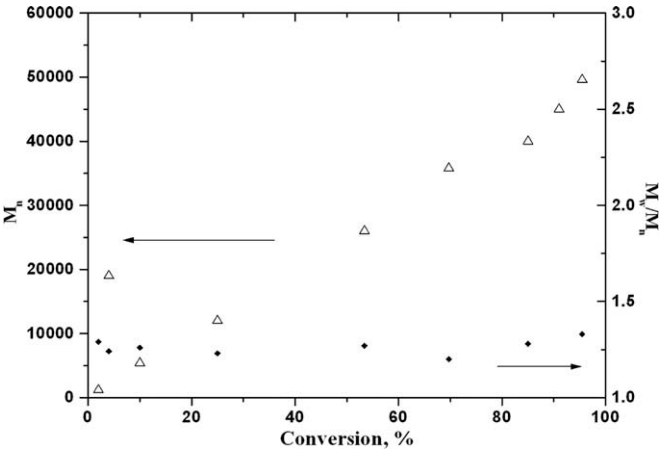

Fig. 3. Evolution of M_n and M_W/M_n as a function of monomer conversion for the polymerization of Star-p$(MeDMA)_3$ at 250/1/2/5 and 60 °C.

Table 1
Conditions and results of ATRP for 21ACSPs.

Samples	$[M]_0/[I]_0/[Cu]_0/[L]_0$[a]	Temp. (°C)	Time (h)	Conv. (%)[b]	M_n,th[c]	M_n,exp	M_W/M_n	Charge density ($\times10^{-3}$ eq/g)[d]
Star-p(MeDMA)1	20/1/2/5	60	6	95.7	8060	6880	1.15	5.10
Star-p(MeDMA)2	100/1/2/5	60	6	92.8	23220	21450	1.26	5.98
Star-p(MeDMA)3	250/1/2/5	60	6	95.5	53650	49600	1.30	6.12
Star-p(HAEAPMA)	100/1/2/5	60	6	90.6	22200	20800	1.18	0.262
Star-p(DMAEMA)	100/1/2/5	60	6	91.2	18220	17400	1.19	0.305
Star-p(DMAPMAm)	100/1/2/5	60	6	93.0	19800	18700	1.22	0.440

[a] $[M]_0$, $[I]_0$, $[Cu]_0$ and $[L]_0$ represent initial monomer concentration, 21Br-β-CD, Cu(I)Br and ligand (2,2′-bipyridine), respectively.
[b] Determined by ^1H NMR.
[c] Calculated from the $[I]_0$ and monomer conversion.
[d] Tested without pH adjustment.

zation process (see Fig. 2). The maintaining of free radical concentration constant was affected by three aspects: (1) the well-defined and extended configuration of star chains. In the process of ATRP, the initiation sites, i.e., $-(CH_3)_2Br$, are supposed to locate on the outer surface of the initiator core. However, the initiation site could be entangled into the internal of the star due to the substantial increasing of the star branch chain length if the reaction is out of control. As a result, the initiation of further polymerization becomes impossible (Li et al., 2005). The unexpected increasing of star branches can be avoided by the using of NMP/water co-solvent as addressed above, and thus helping to keep the radical concentration constant. (2) The side reactions due to the polar polymerization medium (water). It has been found that the poor control of the aqueous ATRP of hydrophilic methacrylates could be brought by the side reactions in the water because water can displace the halide ligand from the copper(I)-mediated ATRP catalyst and deteriorate the stability of the living radicals (Tsarevsky, Pintauer, & Matyjaszewski, 2004). The decreasing amount of water medium in this system is beneficial to this aspect. (3) Coupling termination between star polymers. In our previous study using gel permeation chromatography (GPC), we found that prolonged polymerization at a high temperature (13.5 h at 90 °C) resulted in a M_W doubling the designed value. We also found some splits or small peaks corresponding to high M_W in the trace chromatography of GPC in some cases.

The phenomena above indicated the importance of controlling the coupling termination in synthesizing well-defined star polymers. The homogeneous system provided by NMP/water solvent made it possible to synthesize the 21ACSPs at a lower temperature and potentially eliminated the combination termination of free radicals among star polymer chains.

As can be seen in Fig. 3, in the range up to 95% conversion, the M_n values increased almost linearly with increasing conversion and the polydispersity remained relatively constant. Otherwise, there could be a sharp increasing point of the M_n as well as for the polydispersity if a large amount of coupling termination happened. The result indicated that the polymerization proceeded in a good living protocol and the side reactions such as termination and transfer reactions were thus negligible.

3.2. 21ACSPs/DNA polyplexes

The zeta-potential of the polyplexes was determined as a function of N/P ratio and the results are presented in Fig. 4. It is well known that the surface charge of the polymer/DNA polyplex has great impact on its biological functions: usually a high surface zeta-potential makes it easier for the polyplexes to be taken into the cells by endocytosis (Rémy-Kristensen et al., 2001), resulting in high transfection efficiency, especially in vitro (Takeuchi et al., 1996). However, it may also bring some undesired effects, e.g., non-selectivity to any cells when applied in vivo (Ooya et al., 2006) and higher cytotoxicity (Ogris, Brunner, Schüller, Kircheis, & Wagner, 1999). Thus it would be desirable if we can control the zeta-potential of the DNA polyplexes in a wide range. Star-p$(MeDMA)_2$ was chosen to compare with other 21ACSPs in order to keep the same monomer/initiator ratio and similar molecular weight. As can be seen in Fig. 4, Star-p$(MeDMA)_2$/DNA complex has the highest zeta-potential value at each N/P ratio. This should be due to its high charge density contributed from quaternary ammonium groups as we presented in Table 1. All the 21ACSPs are capable of forming polyplexes with DNA and the zeta-potentials of these polyplexes rang from negative, slightly positive and relatively strong positive, depending on different N/P ratios.

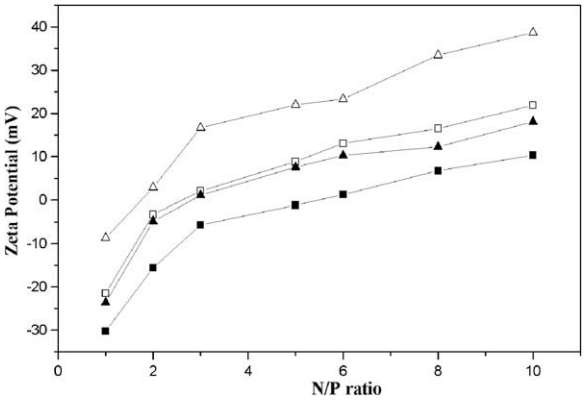

Fig. 4. Zeta-potential of 21ACSPs/DNA polyplexes at different N/P ratios (■: Star-p(HAEAPMA)/DNA; □: Star-p(DMAEMA)/DNA; ▲: Star-p(DMAPMAm)/DNA; △: Star-p$(MeDMA)_2$/DNA).

The results above provide useful information for further various transfection applications in vitro or in vivo. Star-p(DMAEMA), Star-(DMAPMAm) and Star-p$(MeDMA)_2$ all can complex DNA and alter the negative zeta-potential surface (about −40 mV) of DNA to positive at a N/P ratio around 3. Star-p(HAEAPMA)/DNA exhibited the weakest capability for this purpose and it needs a N/P ratio of 6 to reverse the surface charge. It seems that 21ACSP converts the surface charge at a higher N/P ratio compared with linear polymers synthesized from a same monomer. For instance, the charge reversal for linear p-(DMAEMA)/DNA and p(DMAPMAm)/DNA occurred at N/P ratio around 2:1 (van de Wetering, Moret, Schuurmans-Nieuwenbroek, van Steenbergen, & Hennink, 1999). As for the star shape, some functional groups, i.e., cationic amino ones, locate in the internal of the star and cannot be utilized to bind with DNA. Only the cationic groups on the peripheral surface can conjugate with DNA; thus it is reasonable that more star polymers are required to convert the surface charge of DNA. However, the excess amino groups in the internal of the star structure might be useful to act as the "proton sponge" for pH neutralization as those in dendrimers (Lee et al., 1996).

Morphological observations on 21ACSP and 21ACSP/DNA polyplexes using AFM are presented in Fig. 5. AFM scanning in a tapping mode was performed for all the 21ACSPs synthesized in this work. A typical AFM image is shown in Fig. 5(a) (Star-p(MeDMA)2), in which the star shape of the polymers is visible but the exact 21 arms of the polymer cannot be identified. This result seems to be conflicted with the design of the 21 star branches. Following the previous method (Li et al., 2005), we hydrolyzed the star polymers under 1% potassium hydroxide for 60 min to cleave the ester linkage between the β-CD core and arms; and then measured the molecular weight and M_W/M_n of the star arms to determined the number of arms. It was proved that the 21ACSPs indeed have 21 arms. Therefor, the failure in distinguishing each arm might be associated with the sample preparation process for AFM. The star polymers based on β-cyclodextrin core should have a three-dimensional structure, attributed to the three-dimensional feature of cyclodextrin. After 21ACSPs were dried on the surface of the mica disk, the 21 arms cannot maintain well extended in three dimensions as in medium. Most of the arms aggregate together thus we can only view a star shape with several arms.

The size of the Star-p$(MeDMA)_2$ is around 1 μm in diameter as shown in Fig. 5(a). However, since the molecular weight of Star-p$(MeDMA)_2$ is only 21,450, this AFM image could result from the aggregates of several star polymers. The aggregation likely occurred when the star polymer was dried on the mica disk prior to being observed under AFM. Apparently, the sample preparation is crucial for acquiring AFM image with high resolution. The original size of plasmid DNA is around 600 nm. After having conjugated with DNA at a ratio of N/P = 6, Star-p$(MeDMA)_2$ condense the DNA to a size of 80–180 nm as shown in the phase image of AFM

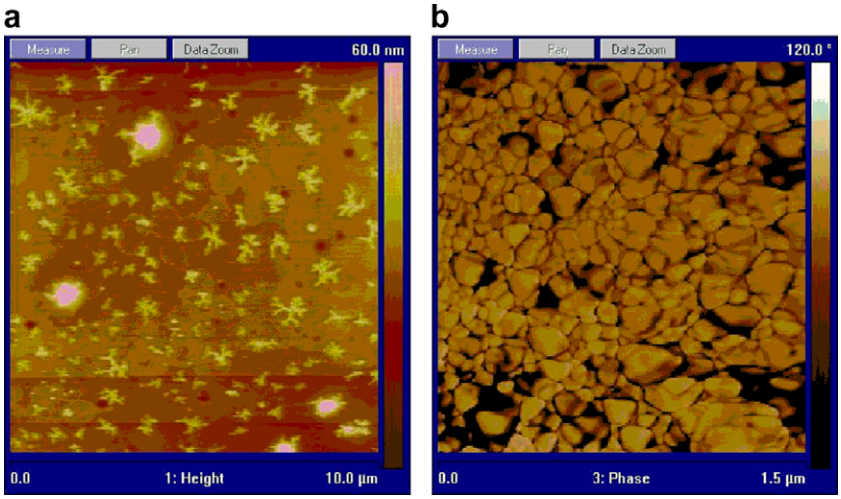

Fig. 5. Atomic force microscopy (AFM) study of 21ACSP and 21ACSP/DNA polyplexes. (a) Star-$(MeDMA)_2$; (b) phase image of Star-$(MeDMA)_2$/DNA polyplexes at N/P = 6.

(Fig. 5b). Dynamic light scattering measurement also provide additional information as the hydrodynamic radii of Star-(MeDMA)2/DNA polyplex at N/P = 6 in PBS (pH 7.4) was 71.0 ± 4.5 nm. The small size of the polyplexes is essential for transferring plasmid DNA into cell membranes and nucleoli.

3.3. In vitro transfection and cytotoxicity

The polyplexes used for cell transfection experiment were formed at N/P = 3, 6, 10 based on the results of zeta-potential measurements. Also, Star-p$(MeDMA)_2$ was selected to maintain the same monomer/initiator ratio and the molecular weight similar to other 21ACSPs. All the polyplexes are either slightly or strongly positively-charged, except for Star-p(HAEAPMA)/DNA at N/P = 3 which is slightly negative. The CHSE-214 cell transfection efficiencies reached by the polyplexes above are presented in Fig. 6 in terms of ng luciferase per mg protein. Combining the results in Table 2 and Fig. 6, it can be found that N/P ratio has profound impact on the cytotoxicity and cell transfection efficiency for each 21ACSP/DNA polyplex. The similar trend, i.e., the transfection efficiency increased but the cell viability decreased with the increase of the N/P ratio, was observed for all polyplexes. This phenomenon is well known, and has been attributed to the increasing amount of free polycation of the polyplexes, as well as to an increase of polyplex uptake (Shuai, Merdan, Unger, Wittmar, & Kissel, 2003; Takeuchi et al., 1996). Meanwhile, the cytotoxicity of all the polyplexes to CHSE-214 cell are lower than that of PEI as shown in Table 2, demonstrating their excellent cell biocompatibility and great potential as DNA vectors. However, additional in vivo toxicity measurements are still necessary in further work for its application in the body.

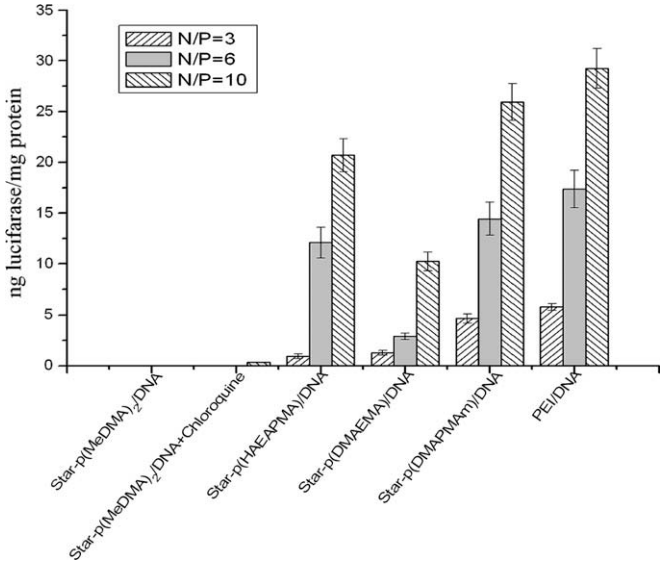

Fig. 6. CHSE-214 cell transfection efficiency by 21ACSP/DNA polyplexes (the negative control sample, i.e., the pCMV-Luc alone, exhibited almost no transfection efficiency).

The different functional groups on the star polymers also play a vital role in their biological performance. The Star-p$(MeDMA)_2$ alone exhibited almost no transfection. This result is not surprising because Star-p$(MeDMA)_2$ was polymerized from a quaternary ammonium monomer, which cannot be further protonated in the acid environment of the endosome. Thus, Star-p$(MeDMA)_2$/DNA polyplexes might be trapped in endosome and cannot be released or destroyed within this organelle prior to releasing. This problem can be somehow addressed by adding an endosomolytic agent, i.e., chloroquine, as shown in the Fig. 6. Clearly, Star-p$(MeDMA)_2$/DNA at N/P = 10 achieved a transfection efficiency about 0.38 ng luciferase/mg protein with the help of chloroquine. The performance of chloroquine in promoting the endosomal escape is consistent with those reported elsewhere (Pouton et al., 1998). However, the efficiency is still significantly lower than that of other 21ACSPs (see Fig. 6). Since chloroquine helped the endosomal escape in this case, its low transfection efficiency was due to the lower affinity between quaternary ammonium groups and DNA under physiological conditions as reported by Reschel, Konak, Oupick, Seymour, and

Table 2
CHSE-214 cell viability in the transfection with 21ACSP/DNA polyplexes.[a]

Polyplexes	Cell viability[b] %		
	N/P = 3	N/P = 6	N/P = 10
Star-p$(MeDMA)_2$/DNA	100.0 ± 2.5	94.0 ± 3.3	84.8 ± 3.0
Star-p$(MeDMA)_2$/DNA + Chloroquine[c]	93.7 ± 3.1	84.2 ± 2.3	77.4 ± 2.5
Star-p(HAEAPMA)/DNA	79.6 ± 3.5	79.5 ± 3.0	77.2 ± 2.0
Star-p(DMAEMA)/DNA	100.0 ± 2.8	92.2 ± 2.5	88.6 ± 2.2
Star-p(DMAPMAm)/DNA	88.1 ± 3.2	78.2 ± 2.6	77.8 ± 2.3
PEI/DNA	75.9 ± 3.0	52.4 ± 2.2	10.7 ± 2.7

[a] Plasmid DNA without star polymers has a cell viability of 90.5 ± 3.5% in the parallel tests.
[b] Calculated based on the protein concentration tested by BCA assay.
[c] Chloroquine was added with the polyplexes.

Ulbrich (2002). In addition, the use of chloroquine decreased the biocompatibility of the polyplexes. As shown in Table 2, Star-p(MeDMA)$_2$/DNA polyplexes have 100%, 94% and 84.8% cell viability at N/P = 3, 6 and 10, respectively. After conjunction with chloroquine the corresponding viabilities were reduced to 93.7%, 84.2% and 77.4%, respectively. On the other hand, Star-p(HAEAPMA)/DNA and Star-p(DMAPMAm)/DNA polyplexes have comparable transfection efficiency to that mediated by PEI, but higher than that of Star-p(DMAEMA)/DNA. The difference should be caused by different pK_a values in physiological pH, which are associated with their structure and functional groups as reported by others (Shuai et al., 2003). It is also of interest that Star-p(HAEAPMA)/DNA at N/P = 3 still exhibited the transfection ability to a certain extent although the polymer has a slightly negative zeta-potential. It is likely attributed to the cationic amino groups in the internal section of the star polymer. Those groups are not bound with DNA while forming the polyplexes but could help the endocytosis process owing to its ability to neutralize the protons under physiological conditions, thus leading to the endosomal escape as dendrimers do (Lee et al., 1996; Tang et al., 1996).

4. Conclusions

In this study, a series of 21 arms cationic star polymers were successfully synthesized by an improved atom transfer radical polymerization performed in the co-solvent of NMP and water at ratio 1:1. The living polymerization proceeded in a well-controlled manner, thus allowing all the 21ACSPs to possess the well-defined structure and the molecular weights close to theoretical ones. The polyplexes formed by 21ACSPs and plasmid DNA were characterized by zeta-potential measurement as well as AFM image. The peripheral surface charges of polyplexes range from negative to positive, depending on different polymer/DNA ratios; and the typical size of polyplexes is within 80–180 nm. 21ACSPs exhibited satisfactory transfection efficiency of plasmid pCMV-Luc to CHSE-214 cells. In addition, the cytotoxicity of the polyplexes is lower than that of PEI during the transfection.

Acknowledgements

Financial supports for this work from the NSERC Canada and the National Natural Science Foundation of China (20704026) are gratefully acknowledged.

References

Bellocq, N. C., Pun, S. H., Jensen, G. S., & Davis, M. E. (2003). Transferrin-containing, cyclodextrin polymer-based particles for tumor-targeted gene delivery. *Bioconjugate Chemistry, 14*, 1122–1132.

Boussif, O., Lezoualc'h, F., Zanta, M. A., Mergny, M. D., Scherman, D., & Demeneix, B. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America, 92*, 7297–7301.

Boussif, O., Zanta, M. A., & Behr, J. P. (1996). Optimized galenics improve in vitro gene transfer with cationic molecules up to 1000-fold. *Gene Therapy, 3*, 1074–1080.

Bremner, K. H., Seymour, L. W., Logan, A., & Read, M. L. (2004). Factors influencing the ability of nuclear localization sequence peptides to enhance nonviral gene delivery. *Bioconjugate Chemistry, 15*, 152–161.

Cho, K. C., Kim, S. H., Jeong, J. H., & Park, T. G. (2005). Folate receptor-mediated gene delivery using folate-poly (ethylene glycol)-poly (L-lysine) conjugate. *Macromolecular Bioscience, 5*, 512–519.

Fischer, D., Bieber, T., Li, Y., Elsasser, H. P., & 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. *Pharmaceutical Research, 16*, 1273–1279.

Georgiou, T. K., Phylactou, L. A., & Patrickios, C. S. (2006). Synthesis, characterization, and evaluation as transfection reagents of ampholytic star copolymers: Effect of star architecture. *Biomacromolecules, 7*, 3505–3512.

Georgiou, T. K., Vamvakaki, M., Patrickios, C. S., Yamasaki, E. N., & Phylactou, L. A. (2004). Nanoscopic cationic methacrylate star homopolymers: Synthesis by group transfer polymerization, characterization and evaluation as transfection reagents. *Biomacromolecules, 5*, 2221–2229.

Kichler, A., Leborgne, C., Coeytaux, E., & Danos, O. (2001). Polyethylenimine-mediated gene delivery: A mechanistic study. *Journal of Gene Medicine, 3*, 135–144.

Kunath, K., von Harpe, A., Fischer, D., & Kissel, T. (2003). Galactose–PEI–DNA complexes for targeted gene delivery: Degree of substitution affects complex size and transfection efficiency. *Journal of Controlled Release, 88*, 159–172.

Lee, R. J., Wang, S., & Low, P. S. (1996). Measurement of endosome pH following folate receptor-mediated endocytosis. *Biochimica et Biophysica Acta. Molecular Cell Research, 1312*, 237–242.

Li, Y., Armes, S. P., Jin, X., & Zhu, S. (2003). Direct synthesis of well-defined quaternized homopolymers and diblock copolymers via ATRP in protic media. *Macromolecules, 36*, 8268–8275.

Li, J., & Loh, X. J. (2008). Cyclodextrin-based supramolecular architectures: Syntheses, structures, and applications for drug and gene delivery. *Advanced Drug Delivery Reviews, 60*, 1000–1017.

Li, J., & Xiao, H. (2005). An efficient synthetic-route to prepare [2,3,6-tri-*O*-(2-bromo-2-methylpropionyl]-β-cyclodextrin). *Tetrahedron Letters, 46*, 2227–2229.

Li, J., Xiao, H., Kim, Y. S., & Lowe, T. L. (2005). Synthesis of water-soluble cationic polymers with star-like structure based on cyclodextrin core via ATRP. *Journal of Polymer Science Part A: Polymer Chemistry, 43*, 6345–6354.

Merdan, T., Kopecek, J., & Kissel, T. (2002). Prospects for cationic polymers in gene and oligonucleotide therapy against cancer. *Advanced Drug Delivery Reviews, 54*, 715–758.

Ogris, M., Brunner, S., Schüller, S., Kircheis, R., & Wagner, E. (1999). PEGylated DNA/transferrin-PEI complexes: Reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Therapy, 6*, 595–605.

Ohno, K., Wong, B., & Haddleton, D. M. (2001). Synthesis of well-defined cyclodextrin-core star polymers. *Journal of Polymer Science Part A: Polymer Chemistry, 39*, 2206–2214.

Ooya, T., Choi, H. S., Yamashita, A., Yui, N., Sugaya, Y., & Kano, A. (2006). Biocleavable polyrotaxane-plasmid DNA polyplex for enhanced gene delivery. *Journal of the American Chemical Society, 128*, 3852–3853.

Opanasopit, P., Petchsangsai, M., Rojanarata, T., Ngawhirunpat, T., Sajomsang, W., & Ruktanonchai, U. (2009). Methylated *N*-(4-*N*,*N*-dimethylaminobenzyl) chitosan as effective gene carriers: Effect of degree of substitution. *Carbohydrate Polymers, 75*, 143–149.

Pack, D. W., Hoffman, A. S., Pun, S., & Stayton, P. S. (2005). Design and development of polymers for gene delivery. *Nature Reviews Drug Discovery, 4*, 581–593.

Pouton, C. W., Lucas, P., Thomas, B. J., Uduehi, A. N., Milroy, D. A., & Moss, S. H. (1998). Polycation-DNA complexes for gene delivery: A comparison of the biopharmaceutical properties of cationic polypeptides and cationic lipids. *Journal of Controlled Release, 53*, 289–299.

Reineke, T. M., & Davis, M. E. (2003). Structural effects of carbohydrate-containing polycations on gene delivery. 1. Carbohydrate size and its distance from charge centers. *Bioconjugate Chemistry, 14*, 247–254.

Rémy-Kristensen, A., Clamme, J. P., Vuilleumier, C., Kuhry, J. G., & Mély, Y. (2001). Role of endocytosis in the transfection of L929 fibroblasts by polyethylenimine/DNA complexes. *Biochimica et Biophysica Acta. Biomembranes, 1514*, 21–32.

Reschel, T., Konak, C., Oupick, D., Seymour, L. W., & Ulbrich, K. (2002). Physical properties and in vitro transfection efficiency of gene delivery vectors based on complexes of DNA with synthetic polycations. *Journal of Controlled Release, 81*, 201–217.

Shen, Y., Li, Q., Tu, J., & Zhu, J. (2009). Synthesis and characterization of low molecular weight hyaluronic acid-based cationic micelles for efficient siRNA delivery. *Carbohydrate Polymers, 77*, 95–104.

Shuai, X., Merdan, T., Unger, F., Wittmar, M., & Kissel, T. (2003). Novel biodegradable ternary copolymers hy-PEI-g-PCL-b-PEG: Synthesis, characterization, and potential as efficient nonviral gene delivery vectors. *Macromolecules, 36*, 5751–5759.

Simms, J. A., & Spinelli, H. J. (1997). Star polymer synthesis. In K. Hatada, T. Kitayama, & O. Vogl (Eds.), *Macromolecular design of polymeric materials* (pp. 379–391). New York: Marcel Dekker.

Takeuchi, K., Ishihara, M., Kawaura, C., Noji, M., Furuno, T., & Nakanishi, M. (1996). Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. *FEBS Letters, 397*, 207–209.

Tang, M. X., Redemann, C. T., & Szoka, F. C. (1996). In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chemistry, 7*, 703–714.

Tsarevsky, N. V., Pintauer, T., & Matyjaszewski, K. (2004). Deactivation efficiency and degree of control over polymerization in ATRP in protic solvents. *Macromolecules, 37*, 9768–9778.

van de Wetering, P., Moret, E. E., Schuurmans-Nieuwenbroek, N. M. E., van Steenbergen, M. J., & Hennink, W. E. (1999). Structure–activity relationships of water-soluble cationic methacrylate/methacrylamide polymers for nonviral gene delivery. *Bioconjugate Chemistry, 10*, 589–597.

Wolfert, M. A., Dash, P. R., Nazarova, O., Oupicky, D., Seymour, L. W., & Smart, S. (1999). Polyelectrolyte vectors for gene delivery: Influence of cationic polymer on biophysical properties of complexes formed with DNA. *Bioconjugate Chemistry, 10*, 993–1004.

Xu, F. J., Zhang, Z. X., Ping, Y., Li, J., Kang, E. T., & Neoh, K. G. (2009). Star-shaped cationic polymers by atom transfer radical polymerization from β-cyclodextrin cores for nonviral gene delivery. *Biomacromolecules, 10*, 285–293.

Yang, C., Li, H., Goh, S. H., & Li, J. (2007). Cationic star polymers consisting of α-cyclodextrin core and oligoethylenimine arms as nonviral gene delivery vectors. *Biomaterials, 28*, 3245–3254.