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# Structural contributions of blocked or grafted poly(2-dimethylaminoethyl methacrylate) on PEGylated polycaprolactone nanoparticles in siRNA delivery

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ABSTRACT

The multiformity in polymer structure and conformation design provides a great potential in improving the gene silencing efficiency of siRNA by polymer vectors. In order to provide information on the polymer design for siRNA delivery, the structural contributions of blocked or grafted poly(2-dimethylaminoethyl methacrylate) on PEGylated polycaprolactone nanoparticles (NPs) in siRNA delivery were studied. Herein, two kinds of self-assembly nanoparticles (NPs) formed by amphiphilic cationic polymers, methoxy poly(ethylene glycol)-block-polycaprolactone-block-poly(2-dimethylaminoethyl methacrylate) (mPEG-PCL-*b*-PDMAEMA, PEC*b*D) and methoxy poly(ethylene glycol)-block-(polycaprolactone-graft-poly(2-dimethylaminoethyl methacrylate)) (mPEG-PCL-*g*-PDMAEMA, PEC*g*D), were used to deliver siRNA for *in vitro* and *in vivo* studies. The physiochemical properties including size and zeta potential of PEC*b*D NPs/siRNA and PEC*g*D NPs/siRNA complexes were characterized. *In vitro* cytotoxicity, cellular uptake and siRNA knockdown efficiency were evaluated in HeLa-Luc cells. The endosome escape and intracellular distribution of PEC*b*D NPs/siRNA and PEC*g*D NPs/siRNA in HeLa-Luc cells were also observed. *In vivo* polymer mediated siRNA delivery and the complexes distribution in isolated organs were studied using mice and tumor-bearing mice. At the same total degree of polymerization (DP) of DMAEMA, PEC*g*D NPs/siRNA complexes possessed higher zeta potentials than PEC*b*D NPs/siRNA complexes (at the same N/P ratio), which may be the reason that PEC*g*D NPs/siRNA complexes can deliver more siRNA into the cytoplasm and lead to higher *in vitro* luciferase and lamin A/C silencing efficiency than PEC*b*D NPs/siRNA complexes. The *in vivo* imaging measurement and histochemical analysis also confirmed that siRNA could be delivered to lungs, livers, pancreas and HeLa-Luc tumors more efficiently by PEC*g*D NPs than PEC*b*D NPs. Meanwhile, the PDMAEMA chains of PEC*g*D could be shortened which provides benefits for clearing. Therefore, PEC*g*D NPs have great potential to be used as efficient non-viral carriers for *in vivo* siRNA delivery.



## 1. Introduction

RNA interference, a natural mechanism of gene silencing in both plant and mammalian cells, has been proven an effective way to inhibit mammalian gene function [1–4]. Small interfering RNA (siRNA), which is generally composed of 21–23 nucleotide (nt) double-stranded RNA (dsRNA) segments and is able to suppress gene expression for therapeutic purposes [5], has already been demonstrated as a potential therapeutic agent *in vivo*. Whereas, there are delivery limitations of naked siRNA *in vivo*, such as: (a) it tends to be degraded by Rnases; (b) naked siRNA can't be transported through the cell membrane freely; and (c) targeting siRNA at a specific organ is problematic [6]. Therefore, there remains a significant challenge for the delivery of siRNA to realize its full therapeutic potential.

To help the active siRNA access the cytoplasm of the target cell, various delivery systems have been developed, which can typically be classified into two groups: viral and non-viral delivery systems. Viral vectors are highly efficient delivery systems for nucleic acids;

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however, the potential of host immune response and the high cost of production limit their applications [7]. Non-viral delivery systems include liposome systems [8,9] and polymer systems. Synthetic cationic polymers have drawn much attention in the past decades because of low immunoreaction *in vivo*, controllable structure and easy production, such as branched or linear polyethylenimine (PEI) and its derivatives [10,11], polyamidoamine (PAMAM) [12,13], poly(L-lysine) (PLL) [14], poly(β-amino ester) (PAE) [15,16] and poly(2-dimethylaminoethyl methacrylate) (PDMAEMA) [17] etc.

The gene silencing efficiency of polymer vectors/siRNA complexes is lower than that of the viral vectors, but the flexibility in conformation design provides great promising potential in improving the siRNA delivery efficiency of polymer vectors to a level comparable to that of viral vectors. In this work, block and graft structural contributions of cationic segments on PEGylated PCL NPs for siRNA delivery were discussed, in order to provide some suggestions in the rational formulation design of polymer vectors.

Our previous research has showed that PEGylated PDMAEMA for DNA vaccine increased the immunogenicity of the intranasal administered DNA vaccine compared to the homopolymer PDMAEMA [18], due to the reduction in the cytotoxicity and prolongation in circulation time [19]. However, on the other hand, PEGylation also reduced gene transfection efficiency *in vitro*. In order to improve the gene transfection efficiency, we tried to introduce hydrophobic segments between PEG and PDMAEMA and prepared methoxy poly(ethylene glycol)-block-polycaprolactone-block-poly(2-dimethylaminoethyl methacrylate) (mPEG-PCL-*b*-PDMAEMA, PEC*b*D) [20] and methoxy poly(ethylene glycol)-block-(polycaprolactone-graft-poly(2-dimethylaminoethyl methacrylate)) (mPEG-PCL-*g*-PDMAEMA, PEC*g*D) [21]. Fortunately, our research results indicated that the introduction of the hydrophobic chains can improve cellular uptake and the DNA transfection efficiency *in vitro* was largely increased [22]. However, the delivery efficiency of PEC*b*D and PEC*g*D *in vivo* had not been studied. Herein, we aimed to resolve (1) if PEC*b*D and PEC*g*D are also suitable for siRNA delivery, since the molecular topography, conformational flexibility and action mechanism of siRNA are completely different from DNA [23], (2) how PEC*b*D and PEC*g*D perform *in vivo* as siRNA vectors, and (3) the distinction between the structural contributions of the blocked and grafted PDMAEMA of PEC*b*D and PEC*g*D. It could be expected that different structures of cationic segments may have different performance in siRNA delivery and present different knockdown efficiencies.

## 2. Materials and methods

### 2.1. Materials

γ-(2-Bromo-2-methylpropionate)-ε-caprolactone (BMPCL) was synthesized as reported previously [24,25]. Methoxy poly(ethylene glycol) ($mPEG_{45}$, $M_n = 2000$), ε-caprolactone (CL), stannous octanoate ($Sn(Oct)_2$), 2-bromoisobutyryl bromide (BIBB), 2-(dimethylamino)ethyl methacrylate (DMAEMA), copper(I) bromide (CuBr) and 2,2-bipyridine (bPy) were purchased from Sigma–Aldrich.

Dulbecco's modified Eagle's medium (DMEM), Lipofectamine 2000 and fetal bovine serum (FBS) were purchased from Invitrogen Corporation (Carlsbad, CA). Ethidium bromide, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and PEI ($M_w = 25$ K) were purchased from Sigma–Aldrich. Agarose was purchased from GEN TECH (Hong Kong, China). NC siRNA was supplied by Suzhou Ribo Life Science Co., Ltd (Suzhou, Jiangsu Province, China), and the sequences were as follows: sense: 5′-Cy5-CCUUGAGGCAUAC UUCAAAdTdT-3′, antisense: 5′-UUUGAAGUAUGCCUCAAGGdTdT-3′. Cy5-labeled siRNA was purchased from Suzhou Ribo Life Science Co., Ltd (Suzhou, Jiangsu Province, China), and the sequences were as follows: sense strand, 5′-UUCUCC-GAACGUGUCACGUdTdT-3′; antisense strand, 5′-ACGUGACACGUUCGGAGAAdTdT-3′. Cy5 fluorophore was labeled at 5′ of the sense strand. Anti-Luc siRNA was purchased from Suzhou Ribo Life Science Co., Ltd (Suzhou, Jiangsu Province, China), and the sequences were as follows: sense: 5′-CCCUAUUCUCCUUCUUCG CdTdT3′, antisense: 5′-GCGAAGAAGGAGAAUAGGGdTdT-3′. siRNA used for silence lamin A/C gene was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China), and the sequences were as follows: sense strand, 5′-CUGGACUUCCAGAA-GAACAdTdT-3′; antisense strand, 5′-UGUUCUUCUGGAAGUCCAGdTdT-3′.

### 2.2. Synthesis of PECbD and PECgD

PEC*b*D was obtained as described previously [20]. In brief, $mPEG_{45}$, CL, and $Sn(Oct)_2$ were added to a nitrogen-purged dry schlenk flask, then the polymerization was carried out at 140 °C for 10 h to obtain mPEG-*b*-PCL-OH. Subsequently, the ATRP macroinitiator mPEG-PCL-Br was prepared by esterification reaction of mPEG-*b*-PCL-OH with BIBB. Finally, mPEG-*b*-PCL-Br, bPy and CuBr were added to a flask purged by three repeated vacuum/nitrogen cycles. After that, DMAEMA was injected into the flask, and the bulk ATRP was performed at 60 °C for 6 h.

PEC*g*D was synthesized as described before [21]. Macroinitiator mPEG-P(CL-co-BMPCL) was prepared by ring-opening polymerization of CL and BMPCL using $mPEG_{45}$ as the initiator, with $Sn(Oct)_2$ as a catalyst. Then, the ATRP polymerization of DMAEMA was performed in bulk at 60 °C for 12 h. The polymers were characterized by $^1$H NMR (Varian Unity-Plus INOVA 500), and the detailed data are listed in Table 1.

### 2.3. Preparation of nanoparticles (NPs) and NPs/siRNA complexes

NPs were prepared using nanoprecipitation technology. For example, 42.2 mg PEC*b*D1 was dissolved in 2 mL THF, and then the solution was added dropwise into 10 mL of deionized water under magnetic stirring. The mixture was stirred at room temperature for 8 h to remove THF, and the final volume was adjusted to 10 mL with deionized water. Then the pH value of the suspension was adjusted to 7.3 by 1.0 M HCl.

NPs/siRNA complexes were prepared *via* electrostatic interactions. For example, to prepare PEC*b*D1/siRNA complexes at N/P = 10, PEC*b*D1 was diluted to 4.2 mg/10 mL in 10 mM DEPC water (pH = 7.3), siRNA was diluted to 0.665 mg/10 mL in 10 mM DEPC water (pH = 7.3). Complexes were prepared by adding 50 μL NPs suspension to equal volume of siRNA solution by pipetting and then incubated for 30 min before characterization and other experiments.

### 2.4. Agarose gel electrophoresis retardation assay

The agarose gel retardation assay was performed as follows: 20 μL of well incubated NPs/siRNA complexes suspension was mixed with 4 μL of 6 × loading buffer (Takara Biotechnology, Dalian, Liaoning Province, China), and then 20 μL of the mixture was loaded onto 2% agarose gel containing 5 μg/mL ethidium bromide. Electrophoresis was carried out at a voltage of 120 V for 20 min in 1 × TAE running buffer. Finally, the results were recorded at UV light wavelength 254 nm with image master VDS thermal imaging system (Bio-Rad, Hercules, CA).

### 2.5. Characterization of particle sizes and zeta potential

The particle sizes and zeta potential of NPs/siRNA complexes were measured using a Zetasizer 3000HS (Malvern Instrument, Inc., Worcestershire, UK) at a wavelength of 633 nm with a constant angle of 173° at room temperature. Various N/P ratios of complexes suspensions containing 4 μg of siRNA were prepared and diluted with 0.8 mL of double-distilled water before characterization.

### 2.6. Cell viability

*In vitro* cytotoxicity of NPs/siRNA complexes was determined by MTT assay. A luciferase stable expression cell line, HeLa-Luc, was used to evaluate the MTT assay. HeLa-Luc cells were seeded in 96-well plates at $1 \times 10^4$ cells per well and subsequently treated with NPs/siRNA complexes containing 0.25 μg NC siRNA. After

**Table 1**
Chemical structure of PEC*b*D and PEC*g*D copolymers.

| Sample | The molecular structure of the polymer[a] | DP (DMAEMA)[b] | $M_n$[c] ($10^4$ g/mol) | PDI[d] |
|---|---|---|---|---|
| PEC*b*D1 | $mPEG_{45}$-$PCL_{30}$-$PDMAEMA_{90}$ | 90 | 1.96 | 1.25 |
| PEC*b*D2 | $mPEG_{45}$-$PCL_{30}$-$PDMAEMA_{120}$ | 130 | 2.58 | 1.28 |
| PEC*g*D1 | $mPEG_{45}$-($PCL_{25}$-co-(BMPCL-*g*-$PDMAEMA_{23}$)$_4$) | 92 | 2.06 | 1.39 |
| PEC*g*D2 | $mPEG_{45}$-($PCL_{25}$-co-(BMPCL-*g*-$PDMAEMA_{33}$)$_4$) | 132 | 2.67 | 1.37 |

[a] The molecular structure of the polymer was determined by $^1$H NMR, the subscripts represent the degrees of polymerization (DP).
[b] The number of DMAEMA per copolymer chain was determined by $^1$H NMR.
[c] The mean molecular weight ($M_n$) of block and graft copolymers was estimated by $^1$H NMR.
[d] PDI = $M_w/M_n$ measured by GPC.