Biodegradable amphiphilic poly(ethylene oxide)-block-polyesters with grafted polyamines as supramolecular nanocarriers for efficient siRNA delivery

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1. Introduction

RNA interference (RNAi) represents a promising gene silencing technology for functional genomics and a potential therapeutic strategy for a variety of genetic diseases [1–3]. The use of small interference RNA (siRNAs) in gene therapy research has surged over the past years following the discovery that the RNAi mechanism of interference RNA (siRNAs) in gene therapy research has surged over various biological events. However, in the absence of safe and effective carriers for in vivo delivery of small interfering RNAs (siRNAs), application of this technology for therapeutic purposes has lagged behind. The objective of this research was to develop promising carriers for siRNA delivery based on degradable poly(ethylene oxide)-block-polyesters containing polycationic side chains on their polyester block. Toward this goal, a novel family of biodegradable poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) based copolymers with polyamine side chains on the PCL block, i.e., PEO-b-PCL with grafted spermine (PEO-b-P(CL-g-SP)), tetaethylenepentamine (PEO-b-P(CL-g-TP)), or N,N-dimethyldipropylenetiamine (PEO-b-P(CL-g-DP)) were synthesized and evaluated for siRNA delivery. The polyamine-grafted PEO-b-PCL polymers, especially PEO-b-P(CL-g-SP), demonstrated comparable toxicity to PEO-b-PCL in vitro. The polymers were able to effectively bind siRNA, self-assemble into micelles, protect siRNA from degradation by nuclease and release complexed siRNA efficiently in the presence of low concentrations of polyamionic heparin. Based on flow cytometry and confocal microscopy, siRNA formulated in PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) micelles showed efficient cellular uptake through endocytosis by MDA435/LCC6 cells transfected with MDR-1, which encodes for the expression of P-glycoprotein (P-gp). The siRNA formulated in PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) micelles demonstrated efficient endosomal escape after cellular uptake. Finally, MDR-1-targeted siRNA formulated in PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) micelles exhibited efficient gene silencing for P-gp expression. The results of this study demonstrated the promise of novel amphiphilic PEO-b-P(CL-g-polyamine) block copolymers for efficient siRNA delivery.

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binding and condensation, self-assembly into poly-ion complex (PIC) micelles with a diameter around 100 nm, avoiding recognition by reticuloendothelial systems (RES), increasing nuclelease resistance and tolerance under physiological conditions [11–15]. However, the safety profile of these polymers containing large polycactionic segments and their non-biodegradable nature in some cases (e.g., PEI containing polymers) remains an obstacle for clinical application. In this regard, development of siRNA carriers based on biomaterials with a more proven safety record is desirable.

Copolymers with PEO as the shell-forming block and polyester as the core-forming block, such as PEO-b-P(ε-caprolactone) (PEO-b-CL), PEO-b-poly(lactide) (PEO-b-PLA) and PEO-b-P(lactide-glycolide) (PEO-b-P(LG)), are more-established biomaterials for drug delivery [16–18]. The biocompatibility of both the PEO and the polyester block has been demonstrated. PEO has been extensively used for coating different pharmaceuticals to modify their pharmacokinetics, increase their safety or lower their immunogenicity [19,20]. Polymers are proven biodegradable and have a history of safe application in absorbable biomedical devices such as sutures [21,22]. However, the lack of cationic moieties in PEO or polyester blocks limits their usefulness for gene or siRNA delivery [23]. In this study, we reported on the synthesis of a novel family of PEO-b-polyester copolymers grafted with short cationic moieties on polyester segments and explored their safety and potential for the formation of PIC micelles for efficient siRNA delivery.

2. Materials and methods

2.1. Materials

Disopropyl amine (99%), benzyl chloroformate (tech. 95%), sodium (in ker- osin), butyl lithium (Bu-Li) in hexane (2.5 M solution), 3,3-diethoxy-1-propanol (DEP), naphthalene, ethylene oxide (EO), branched PEI (25 kDa), N,N-dicyclohexyl carbodiimide (DCC), N-hydroxysuccinimide (NHS), pyrene, spermine (SP), tetraethylenepentamine (TP), and NN-dimethyldipropylethlenetriamine (DP) were purchased from Sigma Chemicals (St. Louis, MO, USA). ε-Caprolactone was purchased from Lancaster Synthesis (Heysham, UK) and distilled by calcium hydride before use. Stannous octoate was purchased from MP Biomedicals Inc. (Eschwege, Germany). Potassium naphthalene solution was prepared by conventional method and the concentration was determined by titration [24]. The scrambled siRNA (Silencer® Negative siRNA and Silencer® FAM™-labeled Negative siRNA) and the anti-MDR-1 siRNA (siRNA) were purchased from Ambion (Austin, TX). Cell culture media RPMI 1640, penicillin–streptomycin, fetal bovine serum, 4-glutamine and HEPES buffer solution (1 M) were purchased from Gibco, Invitrogen Corp (USA). All other chemicals were reagent grade. MDA435/LCC6 cells transfected with MDR-1 overexpressing P-glycoprotein (P-gp) on their membrane, were a gift from the laboratory of Dr. Clarke (Georgetown University Medical School, Washington, DC) [25,26]. Cells were grown as adherent cultures and maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37 °C and 5% CO2.

2.2. Synthesis of PEO-b-PCL with grafted polycation

Poly(ethylene oxide)-b-poly(ε-caprolactone-g-polyamine) (PEO-b-P(CL-g-poly- amine)) block copolymers were prepared from PEO-b-poly(ε-caprolactone) and poly(L-lactide) (PEO-b-PCL). The synthesis of PEO-b-PCL has been described in detail previously [27]. Briefly, PEO-b-poly(ε-caprolactone-g-caprolactone) (PEO- b-PBCL) block copolymer was synthesized by ring-opening polymerization of ε-caprolactone using ε-methoxy-PEO (PEO) as an initiator (Mn = 5000 g mol−1, Mn/Mw = 1.05). Then protective benzyl group of the benzyl-substituted units were removed by the catalytic debenzylation of PEO-b-PBCL in the presence of H2 to obtain PEO-b-PBCL. Then, active ester method was used to attach pendant polycation groups to the polyester section by the amide bond formation using NHS/DCC catalyst system (Scheme 1). In a typical process, PEO-b-PCL (200 mg, ~0.01 mmol) was dissolved in 10 mL of dry THF. After addition of DCC and NHS in THF, the solution was stirred for 2 h until a precipitate was formed. The precipitate was removed by filtration. The polycations, SP, TP, and DP, were dissolved in THF and added drop-wise to the polymer solution. The reaction proceeded for another 24 h under stirring at room temperature. The resulting solution was centrifuged to remove the precipitate followed by evaporation under vacuum to remove the solvents. Methanol (10 mL) was introduced to dissolve the product. The resulting solution was then dialyzed (molecular weight cut-off of 3500 Da) extensively against water and the polymer solution was freeze-dried for further use.

The cytotoxicity of various PEO-b-P(CL-g-polyamine) copolymers against MDR-1 transfected MDA435/LCC6 cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDA435/LCC6 cells (4000 cells/well) were seeded into 96-well plates. After overnight incubation, the culture medium was replaced with 200 µL serial diluted solutions of the polymers, and the wells were incubated for another 48 h. Then, 20 µL of MTT stock solution in phosphate buffered saline (PBS) was added to each well. After 3 h, medium was aspirated and the precipitated formazan was dissolved in 200 µL of DMSO. Cell viability was determined by measuring the optical absorbance differences between 570 and 650 nm using a PowerWaveX340 microplate reader (Bio-TEK Instruments, Inc. VT, USA). The relative cell growth % related to the control containing cell culture medium without polymer was calculated by [Arel/An]×100. All the tests were performed in triplicate. The concentration of drugs leading to 50% cell growth inhibition (IC50) was estimated from the plot of the percentage of viable cells versus log DOX concentration for each treatment.

2.4. Haemolysis assay

The synthesized PEO-b-P(CL-g-polyamine) copolymers, PEI and Triton X-100 (1%, w/v) were dissolved in PBS (pH 7.4). Using blood obtained from a male Sprague-Dawley rat by cardiac puncture, erythrocytes were isolated by centrifugation at 1500 rpm for 10 min.

Scheme 1. Synthetic procedure for the preparation of PEO-b-PCL with grafted SP, TP and DP.
5150 g for 10 min at 4 °C. The cell pellet was resuspended to obtain a 2% (w/v) erythrocyte suspension with pre-chilled PBS, and then added into a 96-well plate (100 μL/well). 100 μL of test samples were then added to the erythrocyte suspension in the multiwell plate which was incubated at 37 °C for 1 h. The supernatant from each sample was removed to a new plate and the absorbance was measured at 550 nm using a microplate reader. The haemolysis % of each polymer was estimated by comparing their absorbance to that from Triton X-100 treatment, which led to 100% haemolysis and was used as the positive control.

2.5. Determination of siRNA binding by gel retardation assay

The siRNA binding ability of the PEo-b-P(CL-g-polyamine) copolymers was analyzed by agarose gel electrophoresis [28]. The PEo-b-P(CL-g-polyamine)/siRNA complexes were prepared by mixing 8 μL of 0.1 w% HEPES buffer (pH 6.5) with 4 μL of negative siRNA (containing 2 μg siRNA) and 8 μL of serially-diluted concentrations of a PEo-b-P(CL-g-polyamine) solution (containing polymers ranging from 1 to 64 μg) and incubated for 30 min at 37 °C. The mixture was incubated for 30–60 min at 37 °C, after which 4 μL of 6× sample buffer (50% glycerol, 1% bromophenol blue, and 1% cylene cyanol FF in TBE buffer) was added, and the samples were loaded onto 2% agarose gels containing 0.05 mg/mL ethidium bromide (EtBr). Electrophoresis was performed at 130 mV and ~52 mA for 15 min, and the resulting gels were photographed under UV-illumination. The pictures were digitized and analyzed with Scion image analysis software to determine the mean density of siRNA bands. The binding percentage was calculated based on the relative intensity of free siRNA band in each well with respect to wells with free siRNA (i.e., in the absence of any polymers). The binding for each polymer was tested at least in 2 independent experiments.

2.6. siRNA release by polyanion competition

The ability of complexes to release siRNA after a challenge with the competing polyanionic heparin was determined as a measure of complex stability [29]. Complexes were prepared at polymer:siRNA mass ratio of 32:1 to ensure complete binding of siRNA by the polymers, and then incubated with 0.78, 1.52, 3.04, 6.08, 12.48, and 24.32 μg of heparin sulfate at 37 °C for 1 h. The solutions were run on agarose gel as described earlier. Results were presented as average of at least 2 independent experiments.

2.7. Serum stability study

To determine the protective role of the polymers against siRNA degradation, PEo-b-P(CL-g-polyamine)/siRNA PIC micelles were prepared at several polymer:siRNA weight ratios, ranging from 4:1 to 32:1 and incubated with 25% fetal bovine serum (FBS) for 24 h at 37 °C. At the same time, free siRNA and PEo(siRNA (1:1 in weight ratio) were incubated with 25% FBS at 37 °C for 24 h as the negative and positive controls, respectively. Samples were then incubated for 1 h with excess of heparin to ensure complete release of siRNA from the formulation. The intact siRNA percentage was then analyzed by agarose gel electrophoresis as described earlier. The results shown represent an average of at least 3 independent experiments.

2.8. Characterization of the self-assembly of PEo-b-P(CL-g-polyamine)/siRNA PIC micelles

A change in the fluorescence excitation spectra of pyrene in the presence of varied concentrations of PEo-b-P(CL-g-polyamine) block copolymers was used to determine the critical micellar concentration (CMC) according to the method described previously [30]. To determine the particle size and zeta-potential, copolymer/siRNA micelles were prepared at 32:1 of copolymer:siRNA weight ratio. PEo-siRNA complex was prepared at 1:1 weight ratio for this analysis. Their average diameters and size distributions were estimated by dynamic light scattering (DLS) using Malvern Zetasizer 3000 at 25 °C. Zeta-potential of the prepared complex was also evaluated by the laser-doppler electrophoresis method using Zetasizer 3000.

2.9. siRNA uptake study by flow cytometry

To assay the ability of polymers to transfer siRNA into MDR-1-transfected MDA435/LCC6 cells, carboxyfluorescein (FAM)-labeled siRNA was formulated in the PIC micelles of different PEo-b-P(CL-g-polyamine) copolymers at 32:1 of polymer/siRNA weight ratio or in PEI at 1:1 weight ratio according to the above-mentioned method. MDA435/LCC6 cells in 6-well plates (5 × 10^5 cells per well) were pulsed with PICs containing 100 μM of siRNA. After incubation for 3 h, the cells were washed with cold PBS, trypsinized, and the uptake of FAM-labeled siRNA was detected by a Becton-Dickinson FACSort™ flowcytometer. The RNAi effect was also observed by confocal microscopy. Toward this, cells grown on coverslips were treated with MDR-1 siRNA/polymer complexes (300 nM) for 48 h at 37 °C, washed with fresh media and incubated with FITC-labeled anti-human P-gp antibody (20 μL) for 30 min at 4 °C. After that, cells were washed three times with cold PBS buffer, and the P-gp level was measured by a Becton–Dickinson FACSort™ flowcytometer. The RNAi effect was also observed by confocal microscopy. Toward this, cells grown on coverslips were treated with MDR-1 siRNA/polymer complexes (300 nM) for 48 h at 37 °C, washed with fresh media and incubated with FITC-labeled anti-human P-gp antibody (20 μL/1 mL) for another 30 min at 4 °C. The cells were then washed three times with PBS, fixed in paraformaldehyde in PBS for 10 min, treated with DAPI for nuclei staining, and examined by confocal microscopy.

![Image](image-url)
3. Results

3.1. Synthesis and characterization of PEO-b-PCL with grafted polyamine groups

The PEO-b-(PCL-g-SP), PEO-b-(PCL-g-TP) and PEO-b-(PCL-g-DP) were synthesized from NHS-activated PEO-b-PCL (Scheme 1). The final structure of copolymer was confirmed by 1H NMR (Fig. 1) and IR (Fig. 2). The characteristics of prepared block copolymers are shown in Table 1. Peaks corresponding to specific polyamine groups of SP, TP and DP were observed at 2.1–3.2 ppm in the 1H NMR spectra, indicating the successful conjugation of polyamine groups to block copolymers (Fig. 1). Based on the intensity ratio of proton peak for the polyamine groups (–NH(CH2)–, δ 2.1–3.2) to that for the PCL segment (OC–(CH2)4–CH2O–, δ 4.05), the polyamine substitution levels of the copolymer were calculated at ~48, ~50 and ~80% for PEO-b-(PCL-g-SP), PEO-b-(PCL-g-TP) and PEO-b-(PCL-g-DP), respectively. Successful synthesis of PEO-b-(PCL-g-polyamine)s was also confirmed by IR spectra (Fig. 2). PEO-b-(PCL-g-SP) and PEO-b-(PCL-g-TP), which contain primary and secondary amine groups in their structures showed N–H stretch at ~3260 and 3400 cm⁻¹, while PEO-b-(PCL-g-DP) which contains secondary and tertiary amine groups, showed N–H stretch at ~3260 cm⁻¹. The CMC of PEO-b-(PCL-g-polyamine) polymers (1.95–3.65 µM) was determined to be significantly higher than that of PEO-b-PCL (0.19 µM), but lower than that of PEO-b-PCL (12.2 µM). Conjugation of the more hydrophobic polyamine DP to PEI-b-PBCL led to a copolymer with a lower CMC value as compared to PEO-b-(PCL-g-SP) and PEO-b-(PCL-g-TP) (Table 1).

3.2. Cytotoxicity and haemolysis study

Cytotoxicity of synthesized PEO-b-(PCL-g-polyamine)s was evaluated in MDR-1 transfected MDA435/LCC6 cells using the MTT assay (Fig. 3A). Compared to PEI (IC50, 6.58 µg/mL), PEO-b-(PCL-g-SP), PEO-b-(PCL-g-TP) and PEO-b-(PCL-g-DP) showed significant lower cytotoxicity against MDA435/LCC6 resistant cancer cells and RBC cells, respectively. The polyamine-grafted copolymers did not display a significant haemolytic activity even at highest polymer concentration.

![Fig. 2. IR spectra of (a) PEO-b-P(CL-g-SP), (b) PEO-b-P(CL-g-TP), (c) PEO-b-P(CL-g-TP) and (d) PEO-b-P(CL-g-DP).](image-url)

![Fig. 3. Cytotoxicity (A) and haemolytic activity (B) of the synthesized PEO-b-P(CL-g-polyamine) against MDA435/LCC6 resistant cancer cells and RBC cells, respectively.](image-url)

### Table 1 Characteristics of PEO-b-P(CL-g-polyamine) polymers and the prepared polymer/siRNA PIC micelles

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Polymer Mw</th>
<th>CMC (µM)</th>
<th>Average diameter (nm)</th>
<th>ζ-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO114-b-P(CL-g-SP)12-6</td>
<td>8300</td>
<td>3.15 ± 0.22</td>
<td>56.8 ± 4.2</td>
<td>2.59 ± 0.35</td>
</tr>
<tr>
<td>PEO114-b-P(CL-g-TP)12-6</td>
<td>8500</td>
<td>3.65 ± 0.18</td>
<td>65.4 ± 4.2</td>
<td>2.27 ± 0.12</td>
</tr>
<tr>
<td>PEO114-b-P(CL-g-DP)12-9</td>
<td>9170</td>
<td>1.95 ± 0.22</td>
<td>90.5 ± 3.2</td>
<td>3.31 ± 0.90</td>
</tr>
</tbody>
</table>

* a The first subscript number stands for the polymerization degree of each block and the second one stands for the number of repeated unit with substituted polyamine groups based on 1H NMR analysis.
* b Determined by 1H NMR.
* c Measured from the onset of a rise in the intensity ratio of peaks at 339 nm to 3260 cm⁻¹ and the second one stands for the number of repeated unit with substituted polyamine groups based on 1H NMR analysis.
3.3. siRNA binding and formation of PIC micelles

Agarose gel electrophoresis was utilized to detect complex formation between the synthesized copolymers and the siRNA. This was based on the disappearance of free siRNA bands in the agarose gels. As expected, the synthesized PEO-b-P(CL-g-polyamine) was capable of effectively binding siRNA, resulting in retardation or disappearance of siRNA bands in agarose gel (Fig. 4A). When the N/P ratios were higher than 10:1, the migration of siRNA was completely retarded for all PEO-b-poly(CL-g-polyamine) copolymers. The binding ability of the polycationic copolymers were not significantly different from each other, but less than that of PEI as indicated by a significant left shift in binding versus N/P ratio plots (Fig. 4B). There were no obvious differences in siRNA binding capacity among the copolymers, except that PEO-b-P(CL-g-DP) showed a slightly lower siRNA binding ability in terms of weight ratio (inserted panel in Fig. 4B). The parent PEO-b-PCL showed very low siRNA binding at all polymer:siRNA weight ratios tested, and PEI showed complete siRNA binding even at very low polymer:siRNA weight ratio (<1).

The amphiphilic block copolymer is known to self-assemble into micelles in aqueous solution when the polymer concentration is above its CMC. The formation of PIC micelles of various PEO-b-P(CL-g-polyamine)s with siRNA was investigated by DLS and \( \zeta \)-potential measurements (Table 1). The average diameters of the PIC micelles ranged from 57 to 91 nm depending on the copolymer structure. The PIC particles formed from the PEI was significantly larger (590 nm). The \( \zeta \)-potential of the PIC micelles from the synthesized

![Figure 4](image-url)

**Fig. 4.** Electrophoretic retardation analysis of siRNA binding by different polymers. The gel results for the individual polymers are shown in (A). Lane numbers in (A) corresponds to different polymer/siRNA weight ratios for PEO-b-P(CL-g-SP) (a), PEO-b-P(CL-g-TP) (b), PEO-b-P(CL-g-DP) (c), and PEO-b-PCL (d): (1) 1:0 (siRNA only), (2) 0.5:1, (3) 1:1, (4) 2:1, (5) 4:1, (6) 8:1, (7) 16:1, and (8) 32:1. In the case of PEI (e), the lane numbers correspond to: (1) 1:0 (siRNA only), (2) 0.0155:1, (3) 0.03125:1, (3) 0.0625:1, (4) 0.125:1, (5) 0.25:1, (6) 0.5:1 and (7) 1:1. The densitometric analysis of the binding results is shown in (B). The inserted panel in (B) shows the percentage of siRNA binding versus polymer:siRNA weight ratio.
copolymers were relatively low (2.3–3.3 mV) as compared to the PIC particles formed from PEI (32.7 mV).

3.4. Release of siRNA from polymer/siRNA PIC micelles with polyamion heparin

The siRNA release from various PICs in the presence of heparin is summarized in Fig. 5. The siRNA release from its complex was dependent on heparin concentration. The ratio of heparin to polymer which leads to 50% of siRNA release (RR50) from the complexes was used as a measure of propensity for dissociation. Accordingly, PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) formed more stable complexes with siRNA as compared to PEO-b-P(CL-g-DP), based on the higher RR50 values for the former copolymers (0.10 and 0.12 µg/µg, heparin:polymer, respectively) as compared to the RR50 value of 0.07 µg/µg, heparin:polymer for PEO-b-P(CL-g-DP). Complete siRNA release from all PIC micelles was observed when the heparin:polymer weight ratio reached 0.2:1. All the PEO-b-(CL-g-polyamine)/siRNA complex micelles showed significantly higher siRNA release than the PEI/siRNA complex micelles (RR50 = 9.22 µg/µg, heparin:polymer), even though the latter was prepared at the very low polymer:siRNA weight ratio of 1:1.

3.5. Protection of siRNA in PIC micelles from serum degradation

The protective effect of the PIC micelles against siRNA degradation was assessed in serum (Fig. 6). Free siRNA was not stable in 25% FBS and it was completely degraded with 24 h incubation. For the PEO-b-P(CL-g-polyamine)/siRNA PIC micelles, even the lowest applied polymer:siRNA ratio (8:1) demonstrated a significant protective effect for siRNA where the percentages of intact siRNA in these PIC micelles reached to ~70%. When the ratio is above 16, siRNA was almost fully recoverable and was protected from serum degradation. The synthesized copolymer did not demonstrate a significant difference in the siRNA protection ability.

3.6. Cellular uptake study

FAM-labeled negative siRNA was used to study cellular uptake of siRNA/polymer PIC micelles or PEI/siRNA complexes. Based on flow cytometry, the cellular uptake of siRNA formulated in various PICs by MDA435/LCC6 cells was in the order of PEI > PEO-b-P(CL-g-TP) > PEO-b-P(CL-g-SP) > PEO-b-P(CL-g-DP) = free siRNA (Fig. 7A and B). The cellular uptake was confirmed with confocal microscopy observation (Fig. 7C). Clear siRNA fluorescence was exclusively observed in cytoplasm when siRNA was formulated in PEO-b-P(CL-g-SP) or PEO-b-P(CL-g-TP) micelles as indicated by the red arrows (Fig. 7C-a and C-b, respectively). siRNA formulated in PEO-b-P(CL-g-DP) micelles (Fig. 7C-c) or siRNA alone (Fig. 7C-d) gave almost no detectable fluorescence in the cells. siRNA formulated in PEI produced large particles with bright fluorescence in cytoplasm as well as in nucleus (Fig. 7C-e). Noticeably, the siRNA appeared to remain in more distinct particles when delivered with the PEI, whereas a more diffuse pattern was evident for the siRNA delivered with the PEO-b-(CL-g-polyamines).

3.7. Endosome/lysosome escape for siRNA/polymer complex micelles

PEO-b-P(CL-g-polyamine)s were designed to have protonatable amino groups with pKa’s around the endosomal pH, so as to introduce high buffering capacity that will facilitate endosomal/lysosomal escape [11]. The intracellular uptake of the PIC micelles by endocytosis and the subsequent endosome/lysosome escape were investigated and compared to PEI/siRNA PIC particles using confocal microscopy. The typical images of cells treated with PEO-b-P(CL-g-TP)/siRNA PIC micelles or PEI/siRNA PIC particles are shown in Fig. 8. At 1 h of incubation, a large fraction of PIC micelles or particles were internalized into the acidic compartments as indicated by the yellow color in the merged fluorescence image (Panel d) of the FAM-labeled siRNA (green, Panel a) and Lysotracker (red, Panel b), indicating that the PIC micelles or PEI particles were internalized into cells by endocytosis to form endosomes/lysosomes. At 3 h of incubation, a definite fraction of siRNA (green) in the cytoplasm was not co-localized with Lysotracker (red) and the green fluorescence became relatively stronger than the red fluorescence, suggesting that this fraction was located in a compartment other than the acidic endosomes/lysosomes or the endosomes/lysosomes were disrupted. PEO-b-P(CL-g-SP)/siRNA PIC micelles showed similar results as PEO-b-P(CL-g-TP)/siRNA micelles did (data not shown). Yellow and green fluorescence were seen at the same slide.

![Fig. 5. siRNA dissociation from complexes by heparin competition. siRNA complexed in PEO-b-P(CL-g-polyamine) with a polymer:siRNA weight ratio of 32:1 and in PEI with a weight ratio of 1:1 were incubated for 1 h at 37 °C with increasing concentrations of polyamionic heparin and amount of complex dissociation was determined assessing free siRNA by agarose gel electrophoresis.](image-url)
even after 1 h of incubation for both PEI/siRNA and PEO-b-P(CL-g-TP)/siRNA complexes (Fig. 8e1 and e2 and f1 and f2) pointing to the efficient and rapid endocytosis and endosome/lysosome escape of both delivery systems. The size of the formed endosomes appeared much larger for PEI delivery system. This is not surprising since the size of endosomes is dependent on the endocytosed particles (570 nm for PEI particles versus 65 nm for PEO-b-P(CL-g-TP)/siRNA and PEI/siRNA. (C) Confocal microscopy images of cells after treatment with FAM-siRNA formulated in (a) PEO-b-P(CL-g-SP), (b) PEO-b-P(CL-g-TP), (c) PEO-b-P(CL-g-DP), (d) HEPES and (e) PEI. The nuclei are stained blue (DAPI) and the internalized siRNA appears as green (FAM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

endosome/lysosome escape at different phases can occur in same single cell and at same time point (Fig. 8e1–e4 and f1–f4).

3.8. MDR-1 siRNA silenced P-gp expression on MDA435/LCC6 cells

The ability of PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) to deliver a functional siRNA was evaluated using MDR-1-targeted siRNA to inhibit P-gp expression in MDA435/LCC6 cells. PEI was used as a control carrier. The relative P-gp expression levels by cells treated with different concentrations of anti-MDR-1 or negative siRNA formulations are shown in Fig. 9. Negative siRNA containing formulations failed to inhibit P-gp expression at all concentrations (Fig. 9A). The silencing of P-gp expression was dependent on the concentration of the functional siRNA (Fig. 9B). 100 nM of siRNA in all formulations didn’t produce significant inhibition in the P-gp expression. MDR-1 siRNA (200 nM) formulated in PEO-b-P(CL-g-SP) or PEO-b-P(CL-g-TP) micelles was as effective as PEI/MDR-1 siRNA (1:1 ratio) to inhibit P-gp expression (~20% of P-gp inhibition). When siRNA concentration was
increased to 300 nM, PEO-b-P(CL-g-TP)/MDR-1 siRNA showed significantly higher inhibition of P-gp expression (~60% of P-gp inhibition) than PEO-b-P(CL-g-SP)/MDR-1 siRNA complex micelles and PEI/siRNA complex (~50% of P-gp inhibition).

The inhibition of P-gp expression mediated by MDR-1 siRNA was further observed by confocal microscopy. Fig. 10 shows the fluorescence images of the cells treated with MDR-1 siRNA or negative siRNA complex (300 nM), and stained with FITC-labeled anti-P-gp monoclonal antibody. PEO-b-P(CL-g-TP) and PEO-b-P(CL-g-TP)/MDR-1 siRNA complex micelles as well as PEI/MDR-1 siRNA complex resulted in weaker fluorescence (green) on the cell membrane than the cells treated with negative siRNA, confirming that P-gp expression on the cell membrane was effectively inhibited by MDR-1 siRNA complex micelles.

4. Discussion

In this study, we grafted different polyamine groups to the PCL block of the amphiphilic PEO-b-PCL copolymer to obtain a novel family of biodegradable and self-associating polymers with potential for in vivo siRNA delivery. Amphiphilic PEO-b-polyesters and PEO-b-poly[(l-amino acid)s (PEO-b-PLAA) represent two traditional polymeric biomaterials for self-assembly into micelles for drug delivery in vivo [31]. PEO-b-PLAAs have demonstrated versatility both in drug and in gene delivery due to the introduction of functional groups in the PLAA block. PEO-b-polyesters such as PEO-b-PCL and PEO-b-PLGA have been of interest in drug delivery because of their excellent long-term safety profiles in clinical application and lower CMC. However, their use in gene delivery was limited due to the absence of cationic moieties on their structure and untailorable polyester backbone. To encapsulate genetic cargoes such as plasmid DNA or siRNA into the nonionic polyester based particulate carriers, complicated procedures or organic solvents that could denature nucleic acids might be used to attain sufficient loading efficiency [32]. However, due to weak condensation, the nucleic acids tend to be released rapidly from the carriers. In addition, the nucleic acids are more susceptible to nuclease attack since they are not typically condensed in these carriers. Attempts have been made to modify the polyester block with cationic residues that can provide anchoring site for genetic cargoes. To date, most of the modifications were focused on introducing cationic blocks (e.g. PEI or PLL) to the end of polyester homo or block copolymers [33–35]. To our best knowledge, modification of the polyester backbone by introducing cationic side groups on ester repeat unit has not been reported. Owing to the biocompatibility of the PEO shell and biodegradability of the PCL core, the PEO-b-P(CL-g-polyamine) PIC micelles prepared in the current study are expected to be safe for in vivo administration. Moreover, the biodegradability of the PCL core along with the presence of short polycations in the PEO-b-P(CL-g-polyamine) PIC micelles are expected to provide the required buffering capacity and osmotic drive required for efficient endosomal escape and expression of the incorporated siRNA.

The developed amphiphilic copolymers were shown to be non-haemolytic and less toxic than PEI against the chosen cancer cell line. They were also shown to be able to effectively bind siRNA, self-assemble into micelles and protect siRNA from degradation by nuclease in serum. The polyamines SP, TP and DP, were grafted to PCL block to provide high density of amine for siRNA binding and protection. However, the substitution is not complete and the presence of unsubstituted carboxyl groups in the PCL block may partly counterbalance their siRNA binding capacity by formation of complexes through electrostatic interactions between the protonated amines and de-protonated carboxyl groups on the PCL block. In addition, grafting of polyamine led to higher CMCs for the copolymers. To assure complete siRNA binding and micelle
shown to form micelle with a particle size of 570 nm with shielding the cationic core charge [36,37]. This is in contrast to PEI/water complexes, which remain intact after cellular internalization [44], a contribution from exposure of primary amines in PEI-b-P(CL-g-SP) or PEI-b-P(CL-g-TP) unimers in increasing the cellular uptake of siRNA can be speculated. Finally, a larger particle size might have attributed to the lower cellular uptake of PEI-b-P(CL-g-SP)/siRNA micelles compared to PEI-b-P(CL-g-SP)/siRNA or PEI-b-P(CL-g-TP)/siRNA micelles.

Another distinctive function of grafting polyamine groups to PCL block is that these polyamine molecules contain protonatable amino groups with different pKs, which are designed to introduce high buffering capacity for membrane disruption that will facilitate endosomal/lysosomal escape [11,45]. Confocal images further revealed that PEI-b-P(CL-g-SP) and PEI-b-P(CL-g-TP) can specifically deliver the siRNA into cytoplasm, whereas siRNA delivered by PEI was found to be in cytoplasm as well as in the nucleus (Fig. 7C). Accumulation of siRNA in the cytoplasm where its target mRNA locates (rather than nucleus) provides another advantage for the developed PEI-b-P(CL-g-polyamine) over PEI for siRNA delivery [46]. The specific cytoplasmic delivery of siRNA might be attributed to greater propensity of siRNA to dissociate when complexed by PEI-b-P(CL-g-polyamine). The nucleus trafficking of genetic cargoes delivered by PEI based carrier was also observed by others [47–49]. PEI with high charge density would efficiently condense siRNA to form tightly compact complexes, which remain intact after cellular internalization, endosomal/lysosomal escape, and may then enter the nucleus together with its siRNA cargo. However, due to lower amine density and the presence of unsubstituted carboxyl group, PEI-b-P(CL-g-SP) or PEI-b-(CL-g-DP) could form loosely compact complex, and the complexed siRNA might dissociate from the polymer before or after endosome escape, limiting its localization to the cytoplasm. The biodegradability of the PCL core may also assist in this process. The different morphology of fluorescence in cells also suggests a possibility for different siRNA compaction leading to changes in siRNA intracellular trafficking for PEI as compared to PEI-b-P(CL-g-polyamine) PIC micelles (Fig. 4A).

The endocytosis and endosome escape of PEI-b-P(CL-g-SP)/siRNA and PEI-b-P(CL-g-TP)/siRNA after cellular uptake were further confirmed by confocal microscopic observations (Fig. 8). Although the theory of proton sponge hypothesis is still arguable [50], it has been extensively used to design effective carriers for delivery of genetic cargoes [11,45]. The pH-sensitive cell membrane disruption of siRNA carriers composed of polyamine has well been evidenced and proved to be directly related to their cellular siRNA delivery efficiency [45,51]. Micellar structures based on PEO-b-PLAA with attached polyamine groups were endowed ability for endosome escape and have displayed impressive gene knockdown activity in vitro [11]. Consistent with these findings, we have seen a correlation between efficient endocytosis, endosomal escape and efficient gene silencing by siRNA micelles (Figs. 8–10).

Multidrug resistance, which is commonly caused by overexpression of P-gp encoded by MDR-1, has been one of the major causes of the failure of chemotherapy in cancer patients [52,53]. Modulations of multidrug resistance by pharmaceutical agents,
antibodies, antisense oligonucleotides, and inhibitors of signal transduction have been pursued either by inhibition of P-gp activity or by inhibition of P-gp expression. The clinical benefit of these approaches remains to be realized, however [54–56]. RNAi mediated gene silencing was shown to be specific and potent, and it has been applied to overcome P-gp mediated MDR in different in vitro models [54,57]. MDR-1 siRNA formulated in PEO-b-P(CL-g-polyamine) micelles would provide better chances for tumor-targeted delivery of siRNA through systemic administration. In this in vitro evaluation, we found that the polymeric formulations mediate siRNA silencing of P-gp in a dose-dependent manner in MDA435/LCC6 cells. PEO-b-P(CL-g-TP)/MDR-1 siRNA (300 nM) showed higher P-gp expression inhibition than PEO-b-P(CL-g-SP)/MDR-1 siRNA (300 nM), which may be caused by the higher cellular uptake of MDR-1 siRNA when formulated in PEO-b-P(CL-g-TP) micelles. It is not surprising that higher cellular uptake of MDR-1 siRNA formulated with PEI did not demonstrate any improved P-gp silencing compared to MDR-1 siRNA formulated in PEO-b-P(CL-g-SP) or PEO-b-P(CL-g-TP) micelles, since siRNA formulated PEO-b-P(CL-g-polyamine) micelles appeared to be more efficiently released from complex micelles after cellular uptake. Despite a high concentration of MDR-1 siRNA (300 nM), the maximum inhibition of P-gp expression was <60% for all the MDR-1 siRNA formulations. The lack of complete inhibition is likely due to the high content of P-gp in the chosen cell line (since these cells are transfected for overexpression of P-gp), and the transient duration of P-gp gene silencing effect [58]. The extent of P-gp inhibition remains to be seen in more primary cell lines that express P-gp [60]. Targeted siRNA delivery to silence P-gp gene expression and evaluation of the effect on sensitivity of MDR cells to anticancer drugs are underway in our research group.

5. Conclusions

In conclusion, we report on the design, synthesis and evaluation of a novel family of PEO-b-polyester based polycationic copolymers and explored their potential for siRNA delivery. We demonstrated that all these amphiphilic polycationic copolymers can effectively bind siRNA, self-assemble into micelles and protect siRNA from degradation by nuclease in serum. PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) micelles in particular can efficiently deliver siRNA into cytoplasm by endocytosis and facilitate endosome escape after cellular uptake. MDR-1-targeted siRNA formulated in PEO-b-P(CL-g-SP) and PEO-b-P(CL-g-TP) exhibited efficient gene silencing for P-gp expression. The synthetic amphiphilic PEO-b-P(CL-g-polyamine) block copolymers present a promising efficient carrier for siRNA delivery especially for systemic administration.

Acknowledgements

This research was funded by research grants from Natural Science and Engineering Research Council of Canada (NSERC) and Canadian Institute of Health Research (CIHR).

Appendix

Figures with essential colour discrimination. Certain figures in this article, particularly Figures 8 and 10, are difficult to interpret in...
References


