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

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ABSTRACT

The RNA interference (RNAi) technology has been successfully used in elucidating mechanisms behind 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)), tetraethylenepentamine (PEO-*b*-P(CL-*g*-TP)), or *N*,*N*-dimethyldipropylenetriamine (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 polyanionic 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 effective 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.

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 gene-specific silencing can be exploited in human disease therapy [4,5]. However, due to the large molecular weight, negative charge of siRNA duplexes, and the susceptibility to enzymatic degradation, the effective cellular uptake and intracellular delivery of siRNA for clinical application represent a major challenge for widespread use of RNAi as a therapeutic modality or even as an investigational tool *in vivo* [6–8].

Successful development of RNAi for clinical application is dependent on the discovery of safe and effective carriers [7,9]. In general, the ideal carrier for siRNA should be able to bind and condense siRNA, provide protection against degradation, specifically direct siRNA to target cells, facilitate its intracellular uptake, escape from endosome/lysosome into cytosol, and finally promote efficient gene silencing. Polymeric carriers have been of interest for siRNA delivery because they could be chemically engineered to meet all or some of these requirements simultaneously [4,10]. Among different polymers designed for siRNA delivery, the micelle assembling block copolymers consisting of poly(ethylene oxide) (PEO) and polycation segment such as polyethylenimine (PEI) and poly(L-lysine) (PLL) have been emerging as promising carriers. These polymers are displaying properties suitable for *in vivo* siRNA delivery, including siRNA

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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 nuclease resistance and tolerance under physiological conditions [11–15]. However, the safety profile of these polymers containing large polycationic segments and their non-biodegradable nature in some cases (e.g., PEI containing polymers) remain 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*-polylactide (PEO-*b*-PLA) and PEO-*b*-P(lactide-*co*-glycolide) (PEO-*b*-PLGA), 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]. Polyesters are proven biodegradable polymers 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

Diisopropyl amine (99%), benzyl chloroformate (tech. 95%), sodium (in kerosin), 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*-dicylcohexyl carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), pyrene, spermine (SP), tetraethlyenepentamine (TP), and *N*,*N*-dimethyldipropylenetriamine (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 (*MDR-1* siRNA) were purchased from Ambion (Austin, TX). Cell culture media RPMI 1640, penicillin–streptomycin, fetal bovine serum, L-glutamine and HEPES buffer solution (1 M) were purchased form GIBCO, Invitrogen Corp (USA). All other chemicals were reagent grade. MDA435/LCC6 cells transfected with *MDR-1* overexpressing P-glycoprotein (P-gp) on their cell 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% CO_2.

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

Poly(ethylene oxide)-*b*-poly(ε-caprolactone-*g*-polyamine) (PEO-*b*-P(CL-*g*-polyamine)) block copolymers were prepared from PEO-*b*-poly(α-carboxyl-ε-caprolactone) (PEO-*b*-PCCL). The synthesis of PEO-*b*-PCCL has been described in detail previously [27]. Briefly, PEO-*b*-poly(α-benzyl carboxylate-ε-caprolactone) (PEO-*b*-PBCL) block copolymer was synthesized by ring-opening polymerization of α-benzylcarboxylate-ε-caprolactone (BCL) using α-methoxy-PEO (PEO) as an initiator ($M_n = 5000\ g\ mol^{-1}$, $M_w/M_n = 1.05$). Then protective benzyl group of the benzyl-substituted units were removed by the catalytic debenzylation of PEO-*b*-PBCL in the presence of H_2 to obtain PEO-*b*-PCCL. Then, active ester method was used to attach pendant polyamine groups to the polyester section by the amide bond formation using NHS/DCC catalyst system (Scheme 1). In a typical process, PEO-*b*-PCCL (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 polyamines, 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.

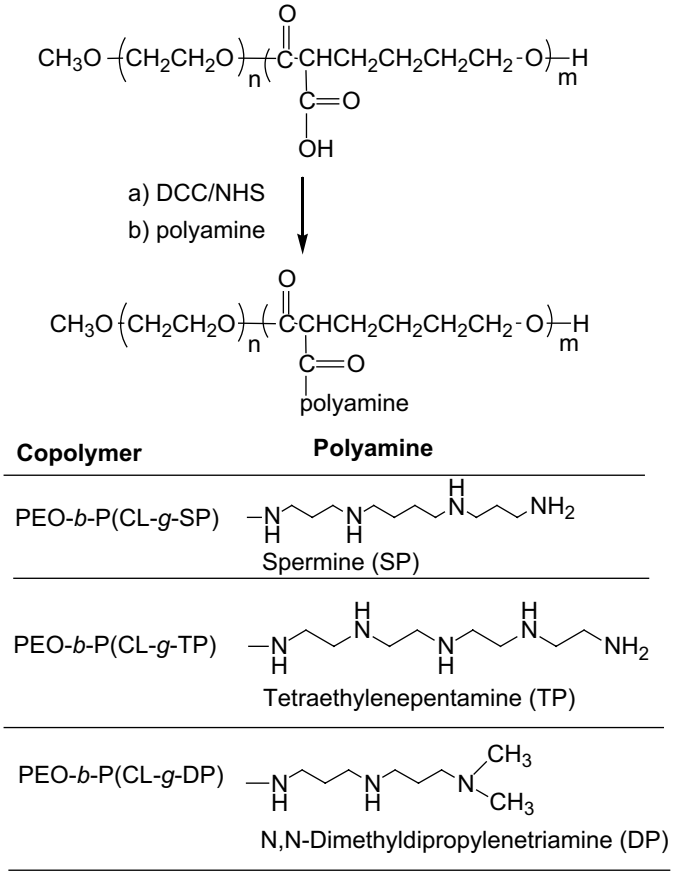
The composition of the reaction products was determined by a 300 MHz ^1H NMR spectroscope (Bruker 300 AM; Billerica MA). The solvent used for ^1H NMR was D_2O for PEO-*b*-P(CL-*g*-SP), PEO-*b*-P(CL-*g*-TP) and $CDCl_3$ for PEO-*b*-P(CL-*g*-DP), respectively. The polyamine substitution level and the molecular weight of the synthesized copolymers were estimated based on peak intensity ratio of the methylene protons from polyamine (–CH_2–NH–) and PEO (–CH_2CH_2O–). The compositions of the synthesized PEO-*b*-P(CL-*g*-polyamine) copolymers were also confirmed by infra-red (IR) spectroscopy using a Nicolet Magna-IR 550 Spectrophotometer (WI, USA).

2.3. Cytotoxicity

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 $[A]_{test}/[A]_{control} \times 100$. All the tests were performed in triplicate. The concentration of drugs leading to 50% cell growth inhibition (IC_{50}) 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



Scheme 1. Synthetic procedure for the preparation of PEO-*b*-PCL with grafted SP, TP and DP.