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Intracellular delivery of VEGF165 encoding gene therapeutic using trifunctional copolymers of ethylene oxide and propylene oxide

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ABSTRACT

New type of copolymers of propylene oxide and ethylene oxide was assessed for promoting delivery of plasmid DNA based gene therapeutics. Lipid-like trifunctional copolymers (TFCs), with both random or diblock structures and relatively low hydrophilic–lipophilic balance, were studied and compared with linear Pluronic™ L61. Structure-dependent relationships for micelle-forming, cytotoxic and hemolytic properties of these copolymers were revealed. The TFC with the mean number of propylene oxide and ethylene oxide units of 83.5 and 24.2, respectively, exhibited relatively low adverse effects in vitro. The latter TFC interacted with plasmid DNA–polyethyleneimine complexes and improved their intracellular delivery. Furthermore, this TFC efficiently promoted the transfection of dermal fibroblasts with VEGF165-encoding Neovasculgen® plasmid DNA, which has been clinically used for the therapeutic angiogenesis. Our findings demonstrated for the first time that TFCs are promising for the polymer-mediated delivery of gene therapeutics.

1. Introduction

Gene therapeutics are promising biologics which have been engineered to treat a host of monogenic and complex diseases including neurodegenerative, cardiovascular and cancer ones [1]. The main engineering platforms for gene therapeutics include synthetic oligonucleotides, viruses and plasmid DNAs (pDNA) [1,2], the two latter types are considered 'pro-drugs' that allow for a template-directed synthesis of a desired peptide within the cell. Compared to viruses, pDNA has a great advantage in clinical application due to its intrinsic safety, low cost and vector capacity. However, pDNA itself suffers from its poor intracellular penetration and stability [3].

Several pDNAs encoding bioactive peptides have been developed and studied as potential drugs in different applications, e.g. for: therapeutic angiogenesis, regeneration of peripheral nerves and bones (see review [4]), cancer treatment [5], and DNA vaccination [6]. However, the improvement of intrinsically low pDNA transfection efficiency remains relevant and unresolved biomedical task. The common approach exploits various cationic lipids and polymers, both of synthetic and natural origin, which bind to, condense and neutralize nucleic acids, thereby improving their stability and cellular pharmacokinetics [2,4].

While cationic carriers of pDNA exhibit a relatively high efficiency in vitro, their gene therapy applications are strictly restricted, due to membrane-damaging and cytotoxic properties of polycations [2,7]. To overcome this problem, new polymeric systems that deliver pDNA, both alone and combined with polycations, have been proposed.

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Ones of these, non-ionogenic amphiphilic copolymers, particularly promise the delivery of small drugs and nucleic acids into target cells [8].

In particular, block copolymers of ethylene oxide (EO) and propylene oxide (PO), known as Pluronics™ or Poloxamers™, were found to increase the transfection efficiency in vitro for pDNA complexes with polyethyleneimine (PEI) [9] and poly(N-ethyl-4-vinylpyridinium) [10]. The possible effects of amphiphilic polymers in pDNA formulation include: the prevention of aggregation of polyplexes in the presence of serum proteins [9]; promotion of endocytosis and/or lysosomal escape [10] and promoter-specific regulation of gene expression [8,11]. The co-injection of 'naked' pDNA and some Pluronics was shown to allow for an enhancement of intramuscular vector expression [11–13]. Similar to Pluronics, another non-ionogenic block copolymer of poly(lactic-co-glycolic acid), PLGA, and polyethylene glycol, PEG (PLGA–PEG–PLGA) improved the transfection process in vivo [14].

While Pluronics are relatively simple linear polyethers which contain two terminal hydroxyl groups, their supramolecular analogs with a higher functionality and new characteristics are being developed [15]. Such polyfunctional polymers are also of particular interest for drug delivery applications in terms of their enhanced capacity for complex formation with biological components. We demonstrated recently that glycerol-based trifunctional block copolymers of EO and PO (TFCs) are promising analogues of Pluronics which exhibit high membranotropic activity and efficiently promote transmembrane transport of anticancer drugs [16]. Here we study the effect of several TFCs on the delivery of pDNA and its complexes into human cells. For the first time we assessed the ability of amphiphilic polymers to promote the delivery of Neovasculgen®, a vascular endothelial growth factor (VEGF) encoding pDNA, which has been recently developed and introduced in Russia by the Human Stem Cells Institute to treat chronic limb ischemia [17,18].

2. Experimental section

2.1. Materials

Bifunctional block copolymer of EO and PO (Pluronic™ L61) was purchased from Sigma–Aldrich. Trifunctional block copolymers of PO and EO (Laprol™ 6003-2B-18, Laprol™ 5003-2-15, Laprol™ 3603-2-12, structural analogs of Voranols, Dow Chemical) were produced by JSC Nizhnekamskneftekhim (Russia).

Branched 25 kDa polyethylenimine (PEI), Hoechst 33342 and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma–Aldrich. TurboFect™ transfection reagent was purchased from Fermentas. NHS-rhodamine was obtained from Pierce. Pyrene, inorganic salts and solvents were produced by Acros Organics. Cell culture reagents were purchased from PAA Laboratories.

Plasmid DNA (pDNA) encoding enhanced green fluorescent protein (pEGFP-N2) was purchased from Clontech. Neovasculgen® pDNA was kindly provided by Dr. Roman Deev, the Human Stem Cells Institute (Russia). This preparation is a recombinant pDNA composed of a transcription regulating site, the minigene encoding VEGF isoform (165 amino acids), a splicing signal, a polyadenylation signal and transcription terminator SV40; molecular weight (MW) 2817092 (4559 b.p.).

Sandwich ELISA (enzyme-linked immunosorbent assay) kit for human VEGF was obtained from Vector-Best (Russia).

2.2. Characterization of pDNA–polymer complexes

pEGFP-N2 plasmid was isolated from transformed *Escherichia coli* overnight culture with the use of Miniprep plasmid DNA isolation kit (Fermentas). The purity and integrity of pDNA was verified by measuring A_{260}/A_{280} ratio and agarose gel electrophoresis. The mass stoichiometry of pDNA–PEI complexes was determined by the agarose gel retardation assay.

Double complexes pDNA–PEI, pDNA–Turbofect and triple complexes pDNA–PEI–TFC were characterized by the dynamic light scattering (DLS) technique on a Zetasizer Nano ZS analyzer (Malvern Instruments). Hydrodynamic diameter and zeta potential of complexes were registered in 50 mM HEPES buffer (pH 7.0) at a working pDNA concentration of 10 μg/mL. Multi-modal (mean number) distribution based on non-negative least squares algorithm was utilized to evaluate DLS data. The measurements were performed in triplicates.

2.3. Determination of critical micelle concentration

The critical micelle concentration (CMC) of TFCs was determined with the use of pyrene probe as described earlier [19]. Briefly, 200 μL of serially diluted polymer solutions in phosphate buffered saline (PBS), pH 7.0, were pipetted into 96-well plate pre-covered with 2.5 nmol of pyrene from methanol solution. The plate was incubated for 1 h at 37 °C under agitation to allow pyrene to dissolve and redistribute into polymeric micelles. Emission spectra of pyrene were detected at RT using an Infinite 200 PRO multimode microplate analyzer (Tecan) in 365–410 nm wavelength range (λ_{ex} 339 nm). CMC was calculated through the relationship between the fluorescence intensity at λ_{max} 373 nm and the logarithm of copolymer concentration [19].

2.4. Cell isolation and culturing

Human skin fibroblasts (HSFs) were isolated from the skin explant according to the conventional protocol [20]. HEK 293 (human embryonic kidney) cells and A549 cells (human lung adenocarcinoma epithelial cells) were obtained from the ATCC collection. HSFs and HEK 293 cells were cultured in the minimum essential medium Eagle (α-MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/mL streptomycin and 100 U/mL penicillin under standard conditions (37 °C, 5% CO_2 atmosphere). A549 cells were grown in the same conditions, but in the Dulbecco's modified Eagle's medium (DMEM). Adhered cells were collected from the culture flask by