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Research paper

Hydroxyl versus permethylated glycopolymers as gene carriers

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ABSTRACT

The main parameters that contribute to non-viral gene delivery are chemical structure and charge distribution. Indeed, saccharide units have been reported to have specific interactions with proteins located in the outer leaflet of the plasma cell membrane that facilitate the cellular internalization of plasmid-DNA vector complexes. In this work, glycopolymers based on statistical copolymers were synthesized through radical copolymerization of a cationic unit, N-ethyl pyrrolidine methacrylamide (EPA), with two styrenic monomers derived from the hydroxylated and permethylated forms of α-glucose. These copolymers were evaluated as possible non-viral gene carriers, and their ability to complex DNA was evaluated. The transfection efficiency and cytocompatibility of the polyplexes, in both fibroblastic and tumoral murine cell lines, was evaluated. Systems derived from α-glucose (GLCSt), over a monomer concentration range of 5–70 mol%, exhibited high toxicity and low transfection efficiency, and were not able to significantly improve on results obtained from positive poly-EPA (PEPA) and polyethyleneimine (PEI) controls. However, systems derived from the permethylated form of α-glucose (MGLCSt), formed stable complexes with DNA or polyplexes, which showed improved transfection efficiency and cytocompatibility in comparison to positive controls. The high transfection efficiency can be clearly attributed to their cytocompatibility, which was notably found to be different for Swiss fibroblasts and B16 melanoma cells, high for Swiss and low for B16. As such, we present permethylated MCLCSt copolymers as good candidates for the possible development of therapies against melanoma.

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1. Introduction

Over recent years, significant efforts have been devoted to the synthesis of novel polymeric cationic vectors [1–4] that could become alternatives to viruses in gene therapy. Such alternatives aim to achieve high transfection efficiency, and overcome virus drawbacks, such as their potential immune responses in therapeutic settings or their high production costs [5]. Most of these cationic polymeric vectors are polyamines, especially polyamines containing secondary and tertiary amino groups that can participate in a phenomenon known as 'proton-sponge' hypothesis for endosomal escape [6–13]. Paradoxically, this net positive charge is also one of their main drawbacks, as it is associated with their high cytotoxicity.

A strategy to modulate the properties and performance of these polyamines may lay in their chemical modification with different entities, of differing biological significance, in order to reduce their positive charge. This type of charge reduction may have consequences for biodistribution, cell-membrane interactions, cytotoxicity, intracellular release, etc. Most of the chemical modifications reported in the literature are based on chain-end conjugation or post-polymerization functionalization. In our previous studies, we have reported an alternative, bottom-up approach, based on the radical copolymerization of the cationizable methacrylamide N-ethyl pyrrolidine methacrylamide (EPA) monomer [14] with neutral and hydrosoluble co-monomers, such as dimethylacrylamide (DMA) [15] or hydroxypropyl methacrylamide (HPMA) [16]. The statistical copolymers obtained exhibited enhanced cyto compatibility/transfection efficiency-balance compared to pure poly-EPA.

In a recently published study [17], two complex styrenic structures, derived from the hydroxylated and permethylated forms of the cyclic oligosaccharide β-cyclodextrin (β-CD), were chosen as the neutral co-monomer entities [18–22]. Post polymerization, the copolymers showed significantly improved cytocompatibility

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and transfection efficiency *in vitro* compared to the EPA homopolymer and the positive polyethyleneimine (PEI) control, especially in the case of the copolymers derived from the permethylated form of β-CD.

These positive results may be attributed to the saccharidic nature of these monomers alone, or they may have been influenced by the unique spatial conformation of the oligosaccharidic ring in the β-CD, and their specific interactions with the plasma membrane [23–26]. In this work, as a new approach in an attempt to clarify and understand these previous results, two new styrenic monomers, derived from the hydroxylated and permethylated forms of α-glucose (the structural unit of the CD ring), were synthesized. It should be noted that the use of saccharides with reduced polarity, as is the case for the monomer derived from permethylated α-glucose, opens a new field of study in gene therapy that, to our knowledge, has no precedents in the literature until the last year [27].

This work describes the synthesis and characterization of two series of statistical linear copolymers derived from EPA and two glucose-derivatized monomers (GLCSt and MGLCSt, Scheme 1). These copolymers were evaluated *in vitro* as possible non-viral gene carriers, using two murine cell lines, simultaneously, and under the same conditions. Specifically, fibroblastic Swiss-3T3 and tumoral B16 (melanoma) cell lines were chosen in order to compare the performance (cytocompatibility and transfection efficiency) of the different copolymers. All tests were performed against polyethyleneimine (PEI) and poly-EPA (PEPA) positive controls, considered PEI the synthetic gold standard alternatives to viral gene vectors in industry.

2. Materials and methods

2.1. Materials

N-(2-aminoethyl) pyrrolidine (ABCR), hyperbranched polyethylenimine (PEI, 25 kDa, Aldrich) and triethylamine (Scharlau) were used without further purification. The radical initiator AIBN (2,2′-azobisisobutironitrile, Fluka), was recrystallized in methanol. Methacryloyl chloride (Aldrich) was distilled before use.

The activity of the commercial plasmid pCMV-GLuc (New England Biolabs) was evaluated after transfection using the BioLux© Gaussia Luciferase Assay Kit (New England Biolabs). The plasmid was amplified in *Escherichia coli* (strain BL21, Sigma-Aldrich) and purified by column chromatography (QIAGEN-Mega kit). The purity of the plasmid was determined using UV spectroscopy (E260 nm/E280 nm with ratio of approximately 1.87–1.89 was used in this study).

2.2. Synthesis of monomers and polymers

The synthesis of the cationic monomer N-ethyl pyrrolidine methacrylamide (EPA) followed a previously reported procedure [14].

2.2.1. Synthesis of the styrenic monomer derived from α-Glucose (GLCSt)

To a solution of methyl 6-deoxy-6-azido-α-D-glucopyranoside [28] (1.09 g, 4.97 mmol) in DMF/H_2O (1:1, 156 mL), propargyl 4-vinylbenzyl ether (1.56 g, 9.86 mmol), $CuSO_4 \cdot 5H_2O$ (0.76 g, 2.76 mmol), and sodium L-ascorbate (0.55 mmol, 2.76 mmol), was added in succession. The mixture was stirred at 80 °C for 1 h. Then, the solvent was evaporated under reduced pressure and the residue was extracted with methanol, filtered and concentrated. The residue was purified by column chromatography (EtOAc:MeOH, 10:1 → 4:1) to give GLCSt (1.22 g, 65%) as a solid. M.p., 116–118 °C. $[\alpha]_D$ = +75.4° (*c* 0.9, chloroform). High resolution mass spectroscopy (HRMS) electrospray ionization (ESI) *m*/*z* (%): calc for $C_{19}H_{26}N_3O_6$ $[M+H]^+$, 392.1822; found, 392.1801. ^1H and ^{13}C NMR characterization and elemental analysis can be found in the Supporting Information.

2.2.2. Permethylation of GLCSt to obtain MGLCSt

To a solution of GLCSt (0.53 g, 1.63 mmol) in DMF (8 mL), NaH (60% in mineral oil, 0.61 g, 15.2 mmol) and MeI (0.53 mL, 8.5 mmol) were added. The mixture was stirred at room temperature for 1 h. Then, MeOH (0.5 mL) was added, and the mixture was diluted with CH_2Cl_2 (50 mL) and washed with H_2O (3x). The organic phase was dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography (EtOAc:hexane, 2:1) to give MGLCSt (0.46 g, 78%) as an oil. $[\alpha]_D$ = +82.3° (*c* 1.0, chloroform). HRMS (ESI) *m*/*z* (%): calc. for $C_{22}H_{32}N_3O_6$ $[M+H]^+$, 434.2291; found, 434.2271. ^1H and ^{13}C NMR characterization and elemental analysis can be found in the Supporting Information.

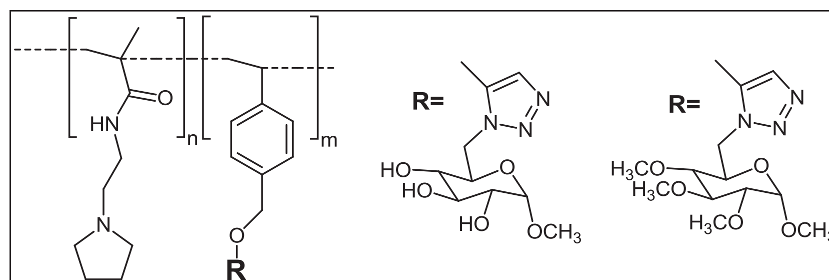
2.2.3. Polymerizations

Poly (EPA-*co*-GLCSt) and poly (EPA-*co*-MGLCSt) copolymers were obtained by free radical polymerization with EPA/(M)GLCSt molar feed ratios of 95:5, 90:10, 80:20, 65:35 and 30:70, at 60 °C for 24 h under an oxygen-free, N_2 atmosphere. The monomer concentration was 1 mol/L and azobisisobutyronitrile (AIBN) (1.5×10^{-2} mol/L) was used as the radical initiator. The copolymerization was carried out using N, N'-dimethyl formamide (DMF) as the solvent. A poly-EPA homopolymer was also synthesized under identical conditions. All polymers were dialyzed against distilled water using Spectra/Por (Spectrum Laboratories Inc.) dialysis membranes with a molecular weight cut-off of 3.5 kDa, before being freeze dried.

2.3. Characterization of the polymer systems

2.3.1. Spectroscopic techniques

All polymer systems were characterized by ^1H Nuclear Magnetic Resonance spectroscopy (^1H-NMR). Spectra were recorded in 5% deuterated chloroform ($CDCl_3$) solution, deuterated methanol (CD_3OD) or deuterated water (D_2O) on a Varian XLR-300 spectrometer, using trimethylsilane (TMS) as the internal standard.



Scheme 1. Chemical structures of the monomers and copolymers studied in this work.

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