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Research Paper

End functionalized polymeric system derived from pyrrolidine provide high transfection efficiency

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ABSTRACT

Chemical architecture and functionality play an important role in the physico-chemical properties of cationic polymers with applications as gene vectors. In this study, linear homopolymers of *N*-ethyl pyrrolidine methacrylamide (EPA), copolymers of EPA with *N*,*N*-dimethylacrylamide (DMA) and oligomers of EPA were synthesized, and the resulting structures were evaluated for their transfection efficiency as non-viral gene vectors. Specifically, polymer species with high and low molecular weights (120–2.6 kDa) and different functionalities (tertiary amines as side chains and primary amine as chain end) were prepared as non-crosslinked, linear homopolymers, copolymers and oligomers, respectively. Polymer/DNA complexes (polyplexes) formation was evaluated by agarose gel electrophoresis, showing that all systems complexed with DNA in all P/N ratios with the exception of the EPA homopolymer. Furthermore, light scattering measurements and transmission electronic microscopy (TEM) showed different size (50–450 nm) and morphology depending on the composition and concentration of the polyplex systems. Cell viability and proliferation after contact with polymer and polyplexes were studied using 3T3 fibroblasts, and the systems showed an excellent biocompatibility at 2 and 4 days. Transfection studies were performed with plasmid Gaussian luciferase kit and were found that the highest transfection efficiency in serum free was obtained with oligomers from the P/N ratio of 1/6 to 1/10. Transfection values of the functionalized oligomers with respect to the control linear poly (dimethylaminoethyl methacrylate) [poly (DMAEMA)] are very interesting in the presence of serum. Haemolysis for these polymers values below 1%, which provide attractive potential applications in gene therapy with these non-toxic readsorbable polymers.

1. Introduction

Gene therapy has become a research area of considerable interest in medicine, pharmaceutics and biotechnology due to the potential for the treatment of chronic diseases and genetic disorders [1]. The basis of gene therapy is the introduction of genes into cells for the production of therapeutic proteins [2]. In this sense, gene delivery systems should be designed to protect the genetic material from premature degradation in the blood stream and to efficiently transfer the therapeutic genes to the target cells. Virus vectors have been widely used as gene carriers as they exhibit good efficiency at delivering DNA to numerous cell lines. However, drawbacks can be attributed to these vectors such as a potential immune response against the transfection system, limitations in the amount and size of the transferred genetic material and high production costs [2]. Synthetic non-viral gene transfer vectors [3], which are free from the risks associated with viral vectors, may represent more suitable gene delivery systems for repetitive use and considerable potential in the gene therapy field. Over the past decade, a large variety of (poly) cationic lipids, liposomes and macromolecules, eventually associated with molecular conjugates for improving cell targeting, cytoplasm delivery and/or nuclear transport, have been used extensively to deliver genes to a large variety of cell lines and tissues [4–10]. These (poly) cationic systems are capable of interacting with anionic DNA, condensing or compacting DNA into small-sized complexes (e.g. polyplexes), neutralizing its negative charges and thus favouring its entry into the cell.

Among the obstacles that non-viral gene transfer vectors have to overcome, the molecular weight is a critical barrier. High molecular weight polymers form extremely stable polyplexes with DNA, which delays the release of the DNA, and the physical shape of the polyplexes is dominated by aggregates [11,12]. In contrast, shorter polycations often display a reduced toxicity including, for example, decreased the complement activation and platelet aggregation in

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comparison with the high molecular weight counterparts [13,14]. As a contribution to this field, some authors have reported on teloplexes (e.g. DNA complexes formulated with telomers of lipids and polymers) as alternative efficient gene transfer agents [15,16]. These telomers (oligomers) [17–20] were obtained from telomerizing one polymerizable acrylamide M monomer, comprising a (poly) amine precursor, in the presence of a hydrophobic long-chain thiol (L-SH). The telomers (oligomers) thus obtained, of formula L-S-(M) *n*-H, showed low degrees of polymerization ($1 \leqslant n < 200$) and, hence, lower molecular weights than compounds prepared by classic polymerization processes ($n \gg 200$). Other authors have described the synthesis of different oligomers by different methods such as a new generation of chitosan-based transfection reagents [21,22,12] or the development of charged sulphonamide-based oligomers [23] having proton buffering capacity and pH-dependent aqueous solubility transition with tuneable pK_a. Moreover, the design of a series of bioreducible, oligoamine-based, linear poly (amido amine)s [24] and the well-defined oligo-[R,S]-3-hydroxybutyrates (OHBs) [25] showed high buffer capacities and lead to high levels of gene expression. Unfortunately, complexes between small, multivalent cations and DNA are not stable under physiological or serum conditions [26] and suffer from concomitant lower transfection efficiency [27]. Consequently, there is a need to develop new compounds, especially non-toxic cationic ones, with characteristics and properties different from those already described.

The present work deals with the synthesis and characterization of a family of linear polymers derived from pyrrolidine and their evaluation as possible carriers for gene delivery. For these purposes, linear acrylic homopolymers, copolymers and oligomers were prepared, and their DNA complexation evaluated complexes size, charge and morphology, as well as their biocompatibility (or cell response). Finally, their transfection efficiency in cell culture, with and without serum, was studied, and their blood compatibility was evaluated for those systems showing the highest transfection efficiency.

2. Material and methods

2.1. Materials

N,N-dimethylacrylamide (DMA) (Aldrich) was vacuum distilled. 4,4′-Azobis (4-cyanopentanoic) (ABCP) (Fluka), ammonium persulphate (APS) (Aldrich), cysteamine hydrochloride (CTA) (Aldrich), ninhydrin (Sigma), 2-ethoxyethanol (Sigma), phosphate buffered saline (PBS, pH 7.4) (Sigma), citric acid (Sigma) and stannous chloride (Sigma) were used as received. Ethyl α-bromoisobutyrate (EBr), (1,1,4,7,7-pentamethyl-diethylenetriamine) (PMDTA), L-ascorbic acid (AA), 2-(dimethylamino) ethyl methacrylate (DMAEMA) and copper (II) chloride ($CuCl_2$) were purchased from Sigma.

Linear poly-L-lysine 70,000–150,000 Da (molecular weight) (Sigma) was used as received. Linear poly (dimethylaminoethyl methacrylate), 10 kDa, PDI: 1.097) (poly-DMAEMA) was synthesized in our laboratory [28]. Plasmid DNA (pDNA) activity after transfection was evaluated using Gaussian princeps luciferase (Gluc, New England Biolabs). The plasmid was amplified in *Escherichia coli* (strain DH5α) and purified by column chromatography (QIAGEN-Mega kit, The Netherlands). The purity of the plasmid was determined by UV spectroscopy (E_{260nm}/E_{280nm} ratio around 1.87–1.89 was used in this study) and by agarose gel electrophoresis.

2.2. Synthesis of homopolymers, copolymers and oligomers

The monomer and homopolymer syntheses of *N*-ethyl pyrrolidine methacrylamide (EPA) were described previously [29] The monomers *N*-ethyl pyrrolidine methacrylamide (EPA) and *N,N*-dimethylacrylamide (DMA) were copolymerized at 50 °C under oxygen-free N_2 atmosphere in mixtures of water/isopropanol ([monomer] = 1 mol/L) using APS (1.5×10^{-2} mol/L) as radical initiator. The oligomer synthesis was carried out by the free radical polymerization of *N*-ethyl pyrrolidine methacrylamide (EPA), in the presence of the appropriate amounts of cysteamine hydrochloride, and ABCP was dissolved in 0.1 M aqueous acetic acid (2 mL) [30]. The reaction mixture was placed under inert atmosphere by repeated flushing with nitrogen. The solution was then heated to 50 °C and maintained at this temperature for 1 day. After 24 h, the homopolymer, copolymers and telomers samples were dialysed against water in a Spectra/Por® (Spectrum Laboratories Inc.) using membranes with a molecular weight cut-off 3500 Da and then lyophilized.

The structures of the homopolymer, copolymers and oligomers are presented in Scheme 1.

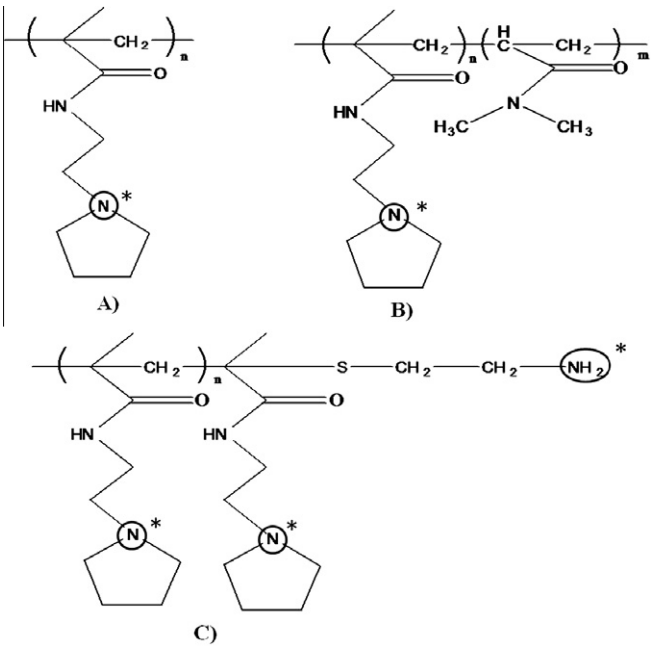
2.3. Characterization of the polymer systems

2.3.1. Spectroscopic techniques

All polymer systems were characterized by ^{1}H Nuclear Magnetic Resonance spectroscopy (NMR). Spectra were recorded in 5% deuterated chloroform ($CDCl_3$) solutions on a Varian XLR-300 spectrometer. IR spectra were recorded at room temperature (25 ± 1 °C) in the mid-infrared range (4000–400 cm^{-1}) using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrometer (FTIR-8300, Shimadzu Europe Ltd., Duisburg, Germany).

2.3.2. Chromatographic techniques

Average molecular weight and molecular weight distributions were determined by size exclusion chromatography (SEC) using polymer solutions (5 mg/mL) in *N,N′*-dimethyl formamide (DMF). Measurements were carried out at 1 mL/min flow with Ultrastyragel columns of 500, 10^4 and 10^5 Å (Polymer Laboratories) at 70 °C and using a differential refractometer as detector. The calibration was performed with poly (styrene) (PS) standards in the range of 2990 and 1,400,000 D and polydispersity values lower than 1.1.



Scheme 1. Chemical structures of the (A) poly-EPA homopolymer, (B) poly (EPA-co-DMA) copolymers and (C) poly-EPA-NH_2 oligomers. *Ionizable groups.