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Preparation of well-defined brush-like block copolymers for gene delivery applications under biorelevant reaction conditions

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ABSTRACT

Well-defined oligo(ethylene glycol) methyl ether methacrylate (OEOMA) based block copolymers with cationic segments composed by *N,N*-(dimethylamino) ethyl methacrylate (DMAEMA) and/or 2-(diisopropylamino) ethyl methacrylate (DPA) were developed under biorelevant reaction conditions. These brush-type copolymers were synthesized through supplemental activator and reducing agent (SARA) atom transfer radical polymerization (ATRP) using sodium dithionite as SARA agent. The synthesis was carried out using an eco-friendly solvent mixture, very low copper catalyst concentration, and mild reaction conditions. The structure of the block copolymers was characterized by size exclusion chromatography (SEC) analysis and ^{1}H nuclear magnetic resonance (NMR) spectroscopy. The pH-dependent protonation of these copolymers enables the efficient complexation with plasmid DNA (pDNA), yielding polyplexes with sizes ranging from 200 up to 700 nm, depending on the molecular weight of the copolymers, composition and concentration used. Agarose gel electrophoresis confirmed the successful pDNA encapsulation. No cytotoxicity effect was observed, even for N/P ratios higher than 50, for human fibroblasts and cervical cancer cell lines cells. The *in vitro* cellular uptake experiments demonstrated that the pDNA-loaded block copolymers were efficiently delivered into nucleus of cervical cancer cells. The polymerization approach, the unique structure of the block copolymers and the efficient DNA encapsulation presented can open new avenues for development of efficient tailor made gene delivery systems under biorelevant conditions.

1. Introduction

Gene therapy is a promising pathway for the treatment of several acquired and inherited genetic diseases, which nowadays still remain impossible to cure. Indeed, gene therapy studies are mostly designed towards cancer research, representing approximately 65% of gene therapy clinical trials worldwide [1]. In particular, tumor suppressor genes, such as p53, are widely explored in gene therapy for cancer treatment [1]. The premise for this approach relies on the fact that cancerous cells lack suitable cell damage regulation, enabling the progression of tumor growth. By inserting into the cells and overexpressing a tumor suppressor gene like p53, it is expected the re-instatement of the mechanisms involved in cancer cell apoptosis [2].

In the past decades, non-viral vectors based on plasmid DNA (pDNA) have become very popular in gene therapy research, because these type of vectors are able to carry larger DNA information, have a simple and low-cost manufacture as well as very low toxicity [3–5]. However, the delivery of pDNA molecules into eukaryotic cells is currently hampered by several hurdles that need to be overcome such as: molecular instability in the blood stream; lack of cell targeting; insufficient cellular uptake; endosomal degradation; immune system activation; among others [6]. Regarding these issues, important research efforts have been made to improve the safety and the efficiency of the gene delivery strategies. Cationic polymers are undeniably the most studied structures for DNA delivery because their positive charges can complex with the negatively charged phosphate groups of pDNA by electrostatic interactions, yielding the so-called polyplexes [6]. The polymer composition, molecular weight (MW), architecture as well as polymer concentration [7] have a major influence on both cytotoxicity and transfection efficiency of the non-viral gene vectors. In the last decades, the development of reversible deactivation radical poly-

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merization (RDRP) methods have turned possible the synthesis of a wide range of well-defined polymers, with controlled and narrow MW distributions, complex architectures and high chain end functionality [8,9]. Amongst RDRP techniques, atom transfer radical polymerization (ATRP) stands out as a very versatile method, as it can be applied to a wide range of monomers under mild reaction conditions [10]. More recently, in pursuing more eco-friendly and less toxic polymerization systems, several ATRP variations have also been proposed [11–15]. Among those, SARA ATRP is particularly effective to synthesize copolymers under biorelevant reaction conditions [16–19].

Weak cationic polyelectrolytes such as DMAEMA have been reported as promising vectors for gene therapy [20]. The tertiary amine side groups are able to efficiently condense the genetic material into nanoparticles [21]. Polymers based on DMAMEA present a transfection efficiency comparable with the "gold standard", branched PEI 25 kDa, without inducing toxicity [22]. Different architectures such as linear copolymers [23–25], star-shaped [26], comb-like [27], branched and other structures [28], have been explored using ATRP techniques. Poly(2-(diisopropylamino)ethyl methacrylate) (PDPA) is another tertiary amine methacrylate with a hydrophilic/hydrophobic transition at pH around 6.2 [29,30], turning this polymer very attractive for biomedical applications. Most, of the reported studies using DPA-based copolymers as non-viral gene delivery systems involve the preparation of micellar nanoparticles [31–33] and pH-sensitive polymersomes for DNA encapsulation [34–36].

Often, the use of cationic polymers is associated to several adverse effects that can be mitigated through the functionalization with poly(ethylene glycol) (PEG) [20,37]. This water soluble polymer provides a steric effect to the polyplex with several benefits; the protection of the nanocarrier payload; prevents serum-induced aggregation; shields the nanocarrier from being recognized from the immune system; reduces the polyplex toxicity; and enhances their *in vivo* stability [38,39]. Recently, the controlled polymerization of PEG based monomers, such as oligo(ethylene glycol) methyl ether methacrylate (OEOMA) proved to be a very efficient tool for polymer PEGylation.[20,40] The architecture and MW of these PEG based monomers influences the size and zeta potential of the PDMAEMA based polyplexes as well as the cellular uptake, transfection efficiency and cell viability [23,41–43]. Stolnik group demonstrated that 'bottle-brush' type PDMAEMA-*b*-POEOMA formed compact complexes with phosphorothioate antisense oligonucleotide, with a long term colloidal stability and high cellular uptake, in contrast, to DMAEMA homopolymer and comb-type statistical DMAEMA-*co*-POEOMA copolymers [42,43]. A similar result was reported by Rudolph and co-workers that synthesized random OEOMA-*co*-DMAEMA copolymers through normal ATRP and studied the influence of the macromolecular structure of OEOMA block in POEOMA-*co*-PDMAEMA copolymers on their ability to condense pDNA [23]. The OEOMA segment prevented gene vector aggregation and did not induce cytotoxicity even at higher pDNA concentrations, but a poor cellular transfection was observed. Recently, the conjugation of polycations with different pK_a values was proposed as an alternative to enhance the transfection efficiency of POEOMA block copolymers [44,45]. Despite the encouraging results of using these non-linear PEG-based monomers in the development of non-viral pDNA carriers, the available literature reports are still scarce. Moreover, all reported polymers were synthesized using multi step laborious and unattractive synthetic routes, such as the use of toxic organic solvents (*e.g.* toluene or tetrahydrofuran) and high concentrations of metal complexes (classical ATRP) [46–48].

In this work, we present an efficient SARA ATRP method to afford well-defined block copolymers composed by OEOMA and two distinct stimuli-responsive tertiary amine methacrylate monomers, DMAEMA and PDA, to be used as gene delivery vectors. The ability of such block copolymers to condense pDNA is evaluated and the physico-chemical properties of the resultant polyplexes are described. *In vitro* experiments are also presented to evaluate the cytotoxicity of the polyplexes into two distinct cell lines (human fibroblasts and cervical cancer cells), and their ability to cellular internalization.

2. Experimental section

2.1. Materials

Sodium dithionite ($Na_2S_2O_4$, 85%, ACROS Organics), copper(II) bromide ($CuBr_2$, 99.9%, Aldrich), ethyl α-bromophenyl acetate (EBPA, 97%, Alfa Aesar), isopropanol (IPA, ACS grade, Fisher Scientific), tetrahydrofuran (THF, ACS grade, Fisher Scientific), deuterated chloroform ($CDCl_3$) (99.8%, Cambridge Isotope Laboratories), deuterium oxide (D_2O) (99.9%, Aldrich) and sodium hydroxide (NaOH) were used as received. 2-(Diisopropylamino)ethyl methacrylate (DPA, 97%, Scientific Polymer Products Inc.), oligo(ethylene oxide) methyl ether methacrylate (OEOMA, 99%, average molecular weight 475, Aldrich) and *N,N*-(dimethylamino) ethyl methacrylate (DMAEMA, 98%, Aldrich) were passed over a column of basic alumina to remove the inhibitor prior to use. Tris(pyridin-2-ylmethyl)amine (TPMA) was synthesized as reported in the literature [49]. Hyper Ladder I (Bioline, London, UK) was used as DNA molecular weight marker. GreenSafe Premium and NZY Maxiprep Kit were purchased from NZYTech (Lisbon, Portugal). Purified water (Milli-Q®, Millipore, resistivity >18 MΩ cm) was obtained by reverse osmosis.

2.2. Methods

A KDS Scientific, Legato 101 syringe pump was used for continuous feeding polymerizations.

High performance size exclusion chromatography (HPSEC) was performed for POEOMA homopolymer samples, using a Viscotek (ViscotekTDAmax) with a differential viscometer (DV), right-angle laser-light scattering (RALLS, Viscotek), and refractive index (RI) detectors. The column set was composed by a PL 10 μm guard column followed by one MIXED-E PLgel column and one MIXED-C PLgel column. Previously filtered THF was used as an eluent at a flow rate of 1.0 mL/min at 30 °C. The samples were filtered through a polytetrafluoroethylene (PTFE) membrane with 0.2 μm pore before injection and the system was calibrated with narrow PS standards. The *dn*/*dc* of POEOMA in THF at 30 °C was determined as 0.072 (for λ = 670 nm) using a Rudolph Research J357 Automatic Refractometer (J357-NDS-670-CC). The M_n^{SEC} and $Đ$ of the synthesized polymers were determined by using a multidetectors calibration (OmniSEC software version: 5.0).

For the block copolymers, the SEC analysis was performed using a system equipped with an online degasser, a refractive index (RI) detector and a set of columns: Shodex OHpak SB-G guard column, OHpak SB-802.5HQ and OHpak SB-804HQ columns. The polymers were eluted at a flow rate of 0.5 mL min^{-1} with 0.1 M Na_2SO_4 (aq) −1 wt% acetic acid −0.02% NaN_3 at 40 °C. Before injection (50 μL) the samples were filtered through a polyester membrane with 0.45 μm pore. The system was calibrated with narrow $Đ$ (M_w/M_n) PEG standards. The number average molecular weight (M_n^{SEC}) and $Đ$ of the synthesized polymers were determined by conventional calibration using Clarity software version 2.8.2.648.

^{1}H nuclear magnetic resonance (NMR) spectra of the reaction mixture and pure copolymers samples were recorded on a Bruker Avance III 400 MHz spectrometer, with a 5 mm TXI triple resonance detection probe, in $CDCl_3$ with tetramethylsilane (TMS) as an inter-