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Research Paper

Effect on *in vitro* cell response of the statistical insertion of *N*-(2-hydroxypropyl) methacrylamide on linear pro-dendronic polyamine's gene carriers

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ABSTRACT

Statistical copolymers of *N*-(2-hydroxypropyl) methacrylamide (HPMA) and the dendronic methacrylic monomer 2-(3-(Bis(2-(diethylamino)ethyl)amino)propanamido)ethyl methacrylate (TEDETAMA, derived from N,N,N′,N′-tetraethyldiethylenetriamine, TEDETA), were synthesized through radical copolymerization and evaluated *in vitro* as non-viral gene carriers. Three copolymers with nominal molar percentages of HPMA of 25%, 50% and 75% were prepared and studied comparatively to the positive controls poly-TEDETAMA and hyperbranched polyethyleneimine (PEI, 25 kDa). Their ability to complex DNA at different N/P molar ratios, from 1/1 up to 8/1, was determined through agarose gel electrophoresis and Dynamic Light Scattering. The resulting complexes (polyplexes) were characterized and evaluated *in vitro* as possible non-viral gene carriers for Swiss-3T3 fibroblasts, using luciferase as reporter gene and a calcein cytocompatibility assay. All the copolymers, except the one with highest HPMA proportion (75% molar) at the lowest N/P ratio, condensed DNA to a particle size between 100 and 300 nm. The copolymers with 25% and 50% molar of HPMA displayed higher transfection efficiency and cytocompatibility than the positive controls poly-TEDETAMA and PEI. A higher proportion of HPMA (75% molar) led to copolymers that displayed very low transfection efficiency, despite their full cytocompatibility even at the highest N/P ratio. These results indicate that the statistical combination of TEDETAMA and HPMA and its fine compositional tuning in the copolymers may fulfill the fine balance of transfection efficiency and cytocompatibility in a superior way to the control poly-TEDETAMA and PEI.

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1. Introduction

In the recent literature there are different examples showing that the copolymerization of neutral *N*-2-(hydroxypropyl) methacrylamide, HPMA, with cationic precursors leads to gene carriers with enhanced performance compared to their homopolymers containing only the cationic component [1–4]. HPMA is a precursor of biocompatible nature, which homopolymer has been used to prepare different soluble drug conjugates with a high residence time in plasma and low tissue distribution compared to other polymer systems [5–10]. Its incorporation to cationic polymeric vectors via a *bottom-up* copolymerization may allow the modulation, in a flexible and tailored way, of the charge density of the polymeric entities and their interaction with the surrounding media. The first of these copolymerization examples was focused on the preparation of block or graft copolymers where the poly-HPMA blocks seemed to play a stabilizing role; these block and graft copolymeric vectors exhibited significantly improved cytocompatibility and transfection efficiency than the pure cationic system [1–3].

Very recently a few examples of the preparation and evaluation of statistical copolymers of HPMA and cationic units, where both structures are distributed randomly along the chains have been reported by us and other authors. These examples have shown also an enhancement of the global performance as gene carriers. Thus, Li et al. evaluated random copolymers of HPMA and guanidinated cationic units as gene carriers and reported a similar performance to PEI [4]. In another more recent example described by us, it was

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found a synergistic effect between HPMA and a methacrylamide bearing a pyrrolidone moiety in the random copolymer structure on the cytocompatibility/transfection balance [11]. It seems that the statistical intercalation of HPMA has a significant effect on the interaction with the nucleic acid and on the interaction of the loaded vector with the media probably due to the decrease and modulation of the local charge's density. It should be noted that statistical copolymers can be prepared in a one-step procedure, more simple that the sequential procedures required for the preparation of block copolymers.

Unlike these two previous examples that statistically combine monocationic units and HPMA, we describe here the copolymerization of HPMA and a multicationic dendronic unit derived from the prodendronic triamine *N*,*N*,*N*′,*N*′-tetraethyldiethylenetriamine (TEDETA), labeled here as TEDETAMA (see Fig. 1). TEDETAMA is a unique unit structure of prodendronic nature; each side moiety bears three aliphatic tertiary amines that are protonated at physiological pH forming polycations with a high intrinsic segmental charge density due to the dendronic nature of the pendant moiety. The linear arrangement of pendant dendronic units, each one carrying three tertiary aliphatic amines mimics, to some extent, an inner block of hyperbranched polyethyleneimine (PEI), a well-known standard polymeric gene vector. It is remarkable that the amines of poly-TEDETAMA exhibit a fully tertiary nature. It is known that tertiary aliphatic amines exhibit a better balance between charged and neutral amines than primary amines, due to their lower pK_a. This leads to reduced cationic character and therefore reduced cytotoxicity and increased buffer capacity, that allows them to participate in the so called 'proton sponge effect' [12–17].

An interesting feature of the homopolymer poly-TEDETAMA, previously reported [18], is its thermosensitivity, exhibiting a lower critical solution temperature (LCST) in aqueous solution which value depends on the pH. Dual sensitivity to pH and temperature has been previously described for structurally related polymers with aliphatic amines in their structure [19–22]. Therefore, the evaluation of the copolymerization with HPMA and the possible LCST shifting to values close to physiological temperature at physiological pH range was a key study of this work.

The homopolymer derived from this pro-dendronic unit has shown previously to be capable of interacting with DNA and transfecting cells *in vitro* with efficiency levels similar to that obtained with PEI vectors [18]. Based on these precedents, this work is devoted to the preparation of different poly-(TEDETAMA-*stat*-HPMA) copolymers, and to their evaluation as possible non-viral vectors, comparatively to PEI and poly-(TEDETAMA), in murine Swiss-3T3 fibroblastic cells *in vitro*. The influence of HPMA on the performance of these linear prodendronic polymers as gene carriers will be evaluated.

2. Materials and methods

2,2′-Azobis-isobutyronitrile (AIBN, Merck) was recrystallized twice from ethanol. Polyethyleneimine (PEI, hyperbranched, M_w = 25 kDa, polydispersity index 2.5) was purchased from Sigma–Aldrich. *N*-(2-hydroxypropyl) methacrylamide (HPMA) was synthesized according to the protocol by Ulbrich et al. [23], which is based on the Schotten–Baumann method, with some modifications [11] that are detailed in the Supplementary Information (SI). The synthesis of 2-(3-(Bis(2-(diethylamino)ethyl) amino)propanamido)ethyl methacrylate (TEDETAMA) has been described previously [18]. Other chemicals purchased from commercial suppliers were of analytical purity or purified by standard techniques.

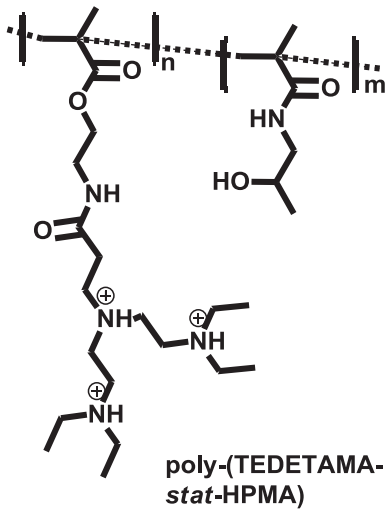

Fig. 1. Structure of the repetitive units of poly-(TEDETAMA-*stat*-HPMA). The multicationic form predominant at physiological pH has been depicted.

The *Gaussia princeps* luciferase plasmid (*pCMV-GLuc*) and the accompanying *BioLux*® *Gaussia Luciferase Assay Kit* were purchased from *New England Biolabs*. This plasmid was amplified using competent *Escherichia coli* (strain BL21, Sigma–Aldrich) and purified with conventional methods. 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).

2.1. Polymer synthesis and characterization

The homo- and copolymerizations were carried out by conventional free radical polymerization at 60 °C, using AIBN ([*I*] = 1.5 × 10^{-2} mol/L) as a radical initiator and a monomer concentration of 1 M; *N*,*N*′-dimethyl formamide (DMF) was used as solvent. Gaseous N_2 was flushed through the polymerizing solution for 30 min. After 24 h, the obtained polymers were extensively washed by dialysis in distilled water and finally the water was removed by a freeze-drying process. The dialysis membranes Spectra/Por® made of regenerated cellulose (*Spectrum Laboratories Inc.*) present a molecular weight cut off 3.5 kDa.

NMR (^{1}H, ^{13}C NMR) spectra were recorded on a 300 MHz (Inova 300 or Bruker 300) and 400 MHz (Inova 400 or Mercury 400) spectrometers, using $CDCl_3$ or D_2O as solvents at room temperature. Chemical shift values are reported in parts per million (δ) relative to Tetramethylsilane (TMS).

The LCST of the polymers was determined by triplicate measuring the optical transmittance of copolymer solutions in aqueous buffer solutions (pH 5, 7, 7.4, 8 and 9) at 500 nm as a function of the temperature. The copolymer concentrations were 2 mg/mL, and the analyses were performed in a UV–visible spectrometer Cary 3 BIO-Varian. The temperature was gradually increased from 25 °C to 90 °C with a heating rate of 1 °C/min. The LCST value was estimated as the temperature at the inflexion point of the absorbance curve against temperature.

The polymers could not be directly analyzed by SEC, neither in DMF (probably due to the low contrast between refractive indices) nor in aqueous pH 3 buffer (the polymer adsorbed in the column) or in THF/triethylamine (the copolymers were not soluble). We therefore degraded the polymers by hydrolyzing the methacrylic ester under harsh conditions (1 Mol/L of NaOH at 60 °C overnight) to analyze the residual poly-(methacrylic acid-*stat*-HPMA) by SEC using DMF as the eluting solvent. The measurements were taken in a Perkin Elmer chromatograph at 0.3 mL/min flow using two

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