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Structure-pDNA complexation and structure-cytotoxicity relationships of PEGylated, cationic aminoethyl-based polyacrylates with tunable topologies

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Abstract

Well-defined, PEGylated aminoethyl-based polyacrylates bearing primary and tertiary ammonium moieties with linear and brush-like PEG topologies were successfully synthesized by successive RAFT copolymerization and amine deprotection. The resulting cationic polymers spontaneously associate with plasmid DNA (pDNA) via efficient condensation to form cationic polymer/pDNA bioconjugates. Effects of polymers structure on the pDNA complexation and on cells viability were assessed by gel retardation assays and MTT assays, respectively. Results demonstrated that bioconjugates with complete pDNA complexation were targeted using equal molar contents of primary and tertiary ammoniums at low N/P ratios (1, 3 and 6) for cationic polymers without PEG, with linear PEG and with brush-like PEG, respectively. Moreover, keeping constant the primary and tertiary ammonium molar composition, linear and brush-like PEGylated aminoethyl-based polyacrylates were less toxic to NIH/3T3 cell line compared to non-PEGylated counterparts. Also, brush-like, PEGylated aminoethyl-based polyacrylates did not induce cytotoxicity even after incubation at high concentration (2 mg.mL⁻¹) and might therefore be envisioned as efficient pDNA vectors for gene therapy applications.

Introduction

Interest in cationic polymers to be employed as non-viral gene delivery systems results from their ability to mediate transfection via the condensation of nucleic acids, to provide protection from enzymatic degradation and to facilitate cellular uptake and endosomal escape.¹ Such a feature is particularly provided by the slightly positive charge of polyplexes which formation is driven by the gain of entropic i.e. the release of small counter ions from both negatively charged nucleic acids and cationic chains during complexation.^{1j} Polyethyleneimine (PEI) is still considered as one of the efficient gene carriers. This success derived from the presence of primary amines that bind to nucleic acid such as DNA and also to the presence of secondary and/or tertiary amines that promote the endosomal escape of the polymer/DNA complexes.² However PEI is rather toxic because of its cationic nature.³ Two main cytotoxic mechanisms for PEI-mediated cell transfection have been established:³⁻⁵ immediate toxicity before internalization and delayed toxicity after internalization. The former is mainly due to the interaction of PEI, that remains in the free form, with charged serum proteins (e.g., albumin) and red blood cells after systemic intravenous administration. Resulting clusters precipitate, adhere to cell membranes causing destabilization and loss of integrity.^{6,7} After internalization, free PEI is restored when DNA cargo is released. The delayed toxicity is associated to cell apoptosis.^{5,6}

Solutions proposed to overcome PEI-related toxicity include the introduction of hydrophobic moieties or its shielding with poly(ethylene oxide) (PEG), also named PEGylation, a well-known polymer used for its non-toxicity and excellent biocompatibility.^{1,8-10} PEG chains hinder the interaction of PEI with blood components thus increasing its circulation time. In addition, PEG offers steric protection of the complexed pDNA against DNase degradation and ensures higher resistance in physiological conditions. Another approach to overcome cytotoxicity issues of cationic polymers relies on the fine control of the amount of primary, secondary, tertiary amines.¹¹

In this context, reversible deactivation radical polymerizations (RDRP) methods, such as nitroxide-mediated polymerization (NMP),¹² atom transfer radical polymerization (ATRP)¹³ and reversible addition-chain transfer (RAFT) polymerization,¹⁴ enabling the synthesis of well-defined, complex macromolecular architecture of controlled composition, appear as essential tools to design cationic polymers with a fine control of molar compositions.

Among the different cationic polymers synthesized via RDRP that have been developed as gene carriers, poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA) has been extensively studied.^{1d,15} For instance, polymethacrylates containing DMAEMA units (tertiary amines) and aminoethyl methacrylate (AEMA) units (primary amines) have been synthesized and the effect of DMAEMA/AEMA molar ratios on transfection efficiency and cytotoxicity have been studied.¹⁶ PEGylation of PDMAEMA/DNA complexes also improved the pharmacokinetic properties by reducing their aggregation with blood components and extending their blood circulation time.^{17,18} Whilst polymers containing DMAEMA units have been extensively studied, their acrylate counterparts based on N,N-dimethylaminoethyl acrylate (DMAEA) were far less encountered. However, PDMAEA is a hydrophilic polymer with great potential for gene delivery applications owing to its capacity to form a polyelectrolyte complex with DNA that can be further disassemble by “charge-shifting” behavior to reduce its cytotoxicity.¹⁹⁻²⁵

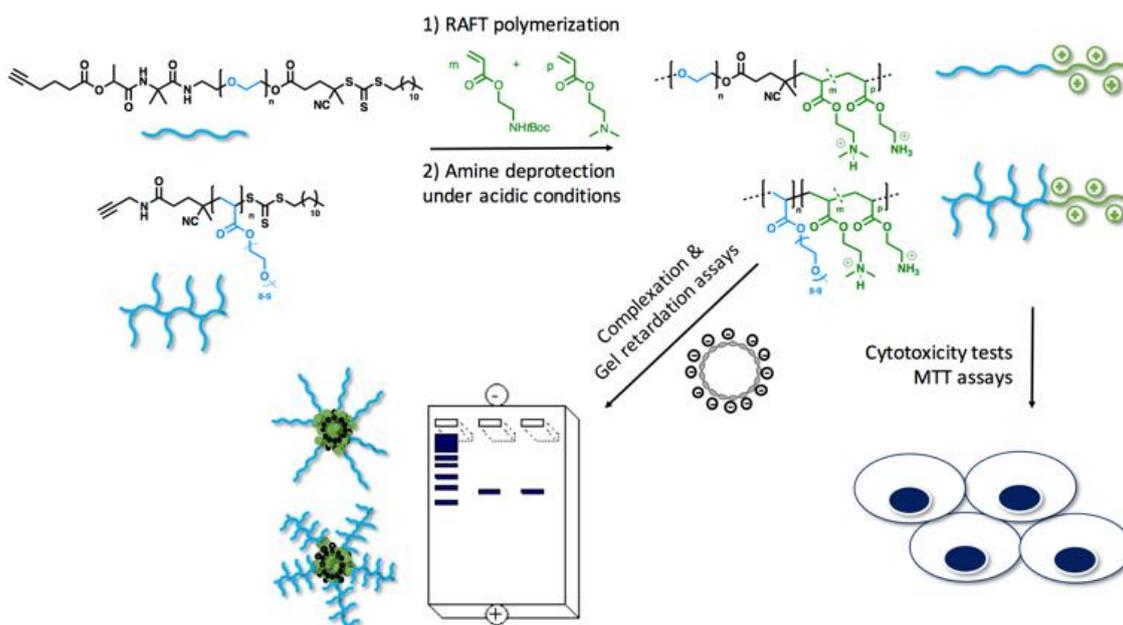
The design of well-defined cationic polyacrylates containing primary and tertiary ammonium pending units was recently reported.²³ Even though these copolymers exhibited “charge-shifting” behavior, the effect of PEGylation and of the topology of the PEG chains linked to the polyacrylate backbone on nucleic acids bioconjugates preparation and cytotoxicity has never been studied. In this work, we have investigated, for the first time, the synthesis of PEGylated, cationic aminoethyl-based polyacrylates bearing protonatable primary and tertiary amines in which the topology of the PEG chains was tuned while maintaining a comparable number of cationic charges. We used RAFT polymerization as a RDRP method given its versatility and the absence of toxicity of polymers generated from trithiocarbonate RAFT agents (Scheme 1).²⁶ Influence of polymer structure on pDNA complexation and cells viability were studied. This study provides insights on the PEGylation of cationic aminoethyl-based polyacrylates and on the nature of PEG moieties on pDNA complexation efficiency and cytotoxicity and pave the way to the design of structurally elaborated, ammonium polyacrylates as potential carriers for nucleic acids (e.g., plasmid DNA, siRNA, etc.).

Results and discussion

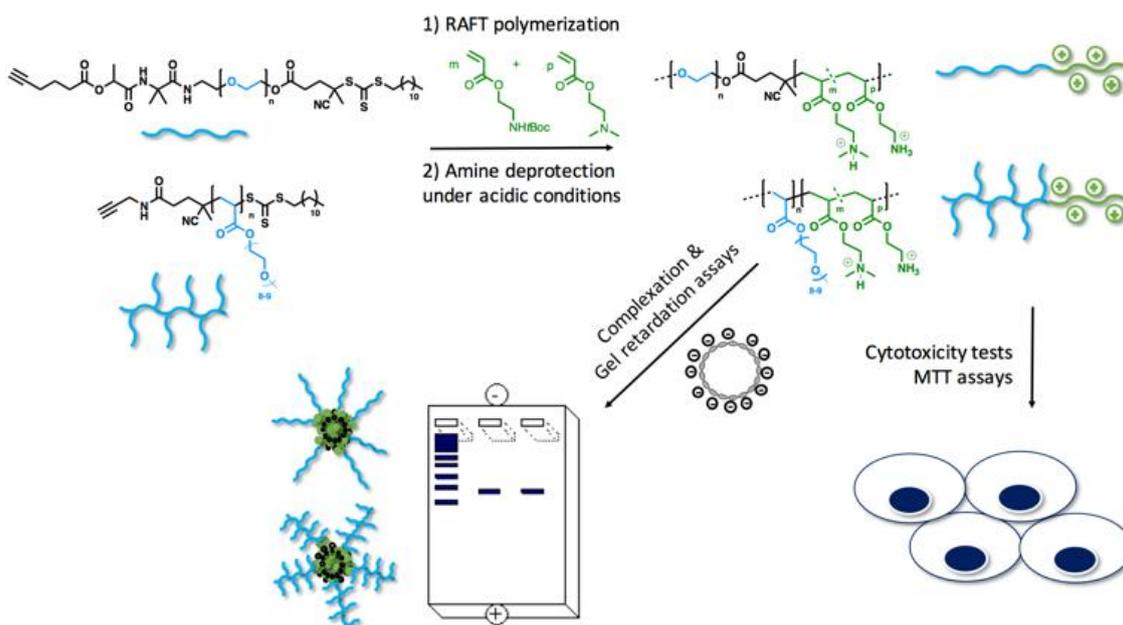
The synthesis of well-defined PEGylated, cationic aminoethyl-based polyacrylates was performed by RAFT copolymerization of aminoethyl-based acrylates (DMAEA and tBocAEA) from PEGylated macromolecular RAFT (macro-RAFT) agents followed by amine deprotection (Scheme 1).

Synthesis of PEGylated macro-RAFT agents

First, linear and brush-like PEG macro-RAFT agents were synthesized. The linear macro-RAFT agent was obtained by a two steps chain-end modification of a commercially available α -amino, ω -hydroxyl-PEG (Scheme 2(A)). The first step is the amine chain-end modification of the α -amino, ω -hydroxyl-PEG by an azlactone (or oxazol-5-one) based linker²⁷ considered as a “masked” activated amino acid that reacts as electrophilic partner with amines and fulfills “click chemistry” expectations.^{29,30} The alkyne chain-end offers the opportunity for further modification. The second step is the addition of the RAFT agent moiety onto linear α -alkynyl, ω -hydroxyl-PEG. Such addition was performed by esterification with 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CDP) in the presence of DCC and DMAP in anhydrous DCM (Scheme 2(A)). The purified product was analyzed by ¹H NMR spectroscopy (Figure S1) and MALDI-TOF mass spectrometry (Figure 1). The ¹H NMR spectrum (Figure S1) shows the characteristic signals including the triplet at 0.88 ppm corresponding to the methyl protons of $\text{SCH}_2(\text{CH}_2)_{10}\text{CH}_3$, the triplet at 3.34 ppm corresponding to the methylene protons of $\text{SCH}_2(\text{CH}_2)_{10}\text{CH}_3$, the triplet at 4.27 ppm corresponding to the methylene protons of $\text{CH}_2\text{CH}_2\text{OC}(=\text{O})$ and the quadruplet at 5.11 ppm corresponding to the methine proton of $\text{CH}(\text{CH}_3)\text{CONH}$ (labeled r, o and e in Figure S1, respectively) highlighting the covalent linkage of the dodecyltrithiocarbonate group on the PEG. The degree of functionalization was calculated to be quantitative.



Scheme 1. Synthesis, pDNA complexation for bioconjugates preparation and cytotoxicity of cationic PEGylated aminoethyl-based polyacrylates obtained by RAFT polymerization.



Scheme 2. Synthetic strategies to target: (A) linear PEG and (B) brush-like PEG macromolecular RAFT agents.

Further analysis of the polymer was performed by MALDI-TOF mass spectrometry (Figure 1). The spectrum shows one series of peaks separated by an average m/z_{exp} equal to 44.087 Da corresponding to the molar mass of the EG repeat unit ($m/z_{\text{calc}} = 44.023$ Da). The peak at $m/z_{\text{exp}} = 3538.038$ Da on the spectrum corresponds to a PEG with 65 repeating units, an alkyne mediated

through azlactone moiety at one chain-end, a hydroxyl moiety at the other chain-end and a sodium atom responsible of the ionization (calculated value $m/z_{\text{calc}} = 3537.991$ Da).

The ^1H NMR spectroscopy and MALDI-TOF mass spectrometry analyses showed the efficiency of the esterification to target well-defined, linear α -alkynyl, ω -dodecyltrithiocarbonate-PEG (referred to as IPEG-CTA) able to react as macro-RAFT agent.

The brush-like PEG macro-RAFT agent (referred to as bPEG-CTA throughout the rest of the manuscript) was synthesized by RAFT polymerization of oligo(ethylene glycol) acrylate (OEGA) using 2-cyano-5-oxo-5-(prop-2-yn-1-ylamino)pentan-2-yl-dodecyltrithiocarbonate (COPYDC) previously synthesized in our laboratory (Scheme 2(B)).²⁸ Although commercially available RAFT agents have already been used to perform the RAFT polymerization of OEGA,³¹⁻³⁴ the COPYDC has never been employed. In our hands, the RAFT polymerization of OEGA using ACVA as the initiator in DMF as the solvent was performed at 70 °C. Given ACVA and DMF are water-soluble and water-miscible, respectively, the resulting polymer is purified by a simple dialysis. A study has been performed using a low $[\text{OEGA}]_0/[\text{COPYDC}]_0/[\text{ACVA}]_0$ initial molar ratio ($=30/1/0.1$) to target well-defined polymers with low and similar molar masses than the IPEG-CTA. Several aliquots were withdrawn at different times intervals during the reaction to follow the characteristics of the polymer by SEC. SEC chromatograms (Figure 2) showed the decrease with time of the refractive index signal intensity of OEGA (unconverted OEGA), a constant shift of the polymer signal toward higher molar masses and unimodal distribution showing no visible sign of radical-radical macromolecular chains coupling dead polymer chain accumulation.

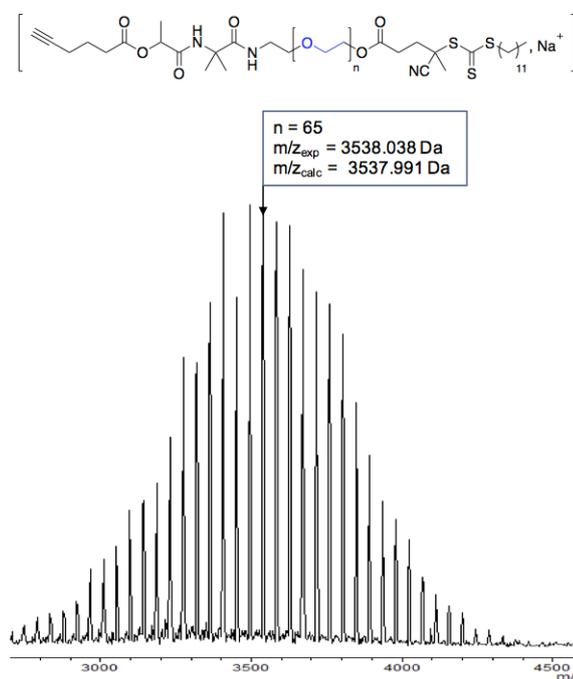


Figure 1. MALDI-TOF mass spectrometry of the α -alkynyl, ω -dodecyltrithiocarbonate-PEG obtained by esterification of the α -alkynyl, ω -hydroxyl-PEG with CDP mediated through DCC and DMAP in DCM at room temperature during 4 days (matrix: DCTB, salt: sodium trifluoroacetate).

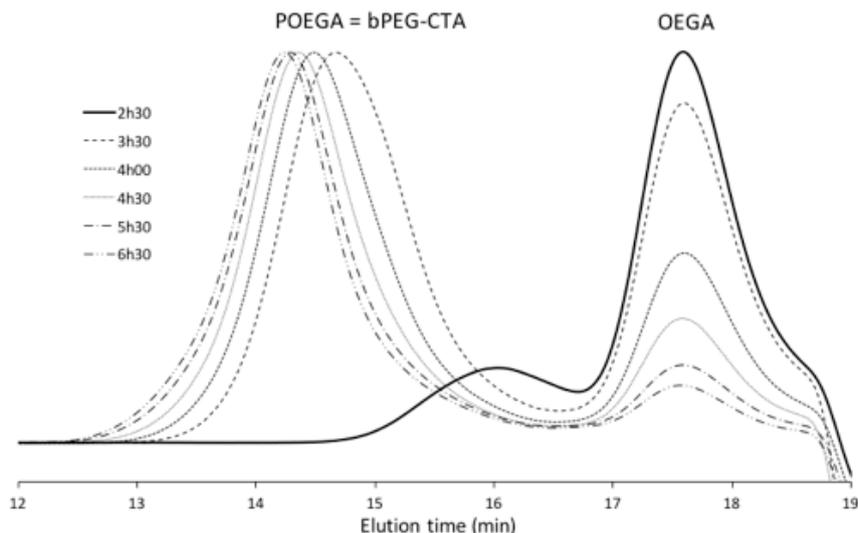


Figure 2. SEC traces (in THF) using RI detection of crude mixtures taken at different time intervals during the RAFT polymerization of OEGA at 70 °C using ACVA as initiator and COPYDC as RAFT agent.

To gain a deeper insight into the PEG topology, a large amount of bPEG-CTA was synthesized keeping the $[OEGA]_0/[COPYDC]_0/[ACVA]_0$ initial molar ratio to 30/1/0.1 and stopping the reaction at relatively low OEGA conversion (52%). The final polymer was purified by dialysis in pure water, lyophilized and analyzed by 1H NMR spectroscopy and SEC using both RI and UV detections. The DP_n of bPEG-CTA was determined by 1H NMR spectroscopy (Figure S2) by comparing the integration areas of methyl protons $SCH_2(CH_2)_{10}CH_3$ of the dodecyl chain at 0.88 ppm and of methylene protons $C(=O)OCH_2CH_2O(CH_2CH_2O)_9$ and $HC\equiv CCH_2NH$ between 4.0 and 4.4 ppm and was found to be 12.

The SEC trace of the purified polymer (RI signal) shows an unimodal and narrow signal giving $M_{n,SEC} = 14\ 700\ g\cdot mol^{-1}$ and $\mathcal{D} = 1.26$ (Figure 3). As the trithiocarbonate group has a strong UV absorption at 309 nm, the SEC analysis was performed using the UV detection fixed at 309 nm (Figure 3). Overlaying RI and UV traces confirmed the chain-end functionalization of the polymer by a trithiocarbonate group. 1H NMR spectroscopy and SEC analyses showed the efficiency of RAFT polymerization to target well-defined bPEG-CTA able to be used as macro-RAFT agents for the copolymerization of aminoethyl acrylates.

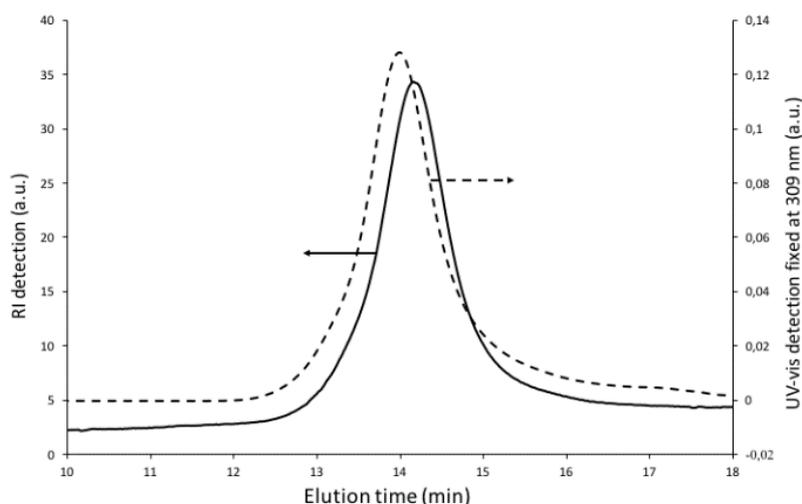


Figure 3. Overlaid SEC traces (in THF) of purified bPEG-CTA using RI detection (solid line) and UV detection at 309 nm (dash line) obtained after the RAFT polymerization of OEGA at 70 °C using ACVA as the initiator and COPYDC as the RAFT agent ($[OEGA]_0/[COPYDC]_0/[ACVA]_0 = 30/1/0.1$).

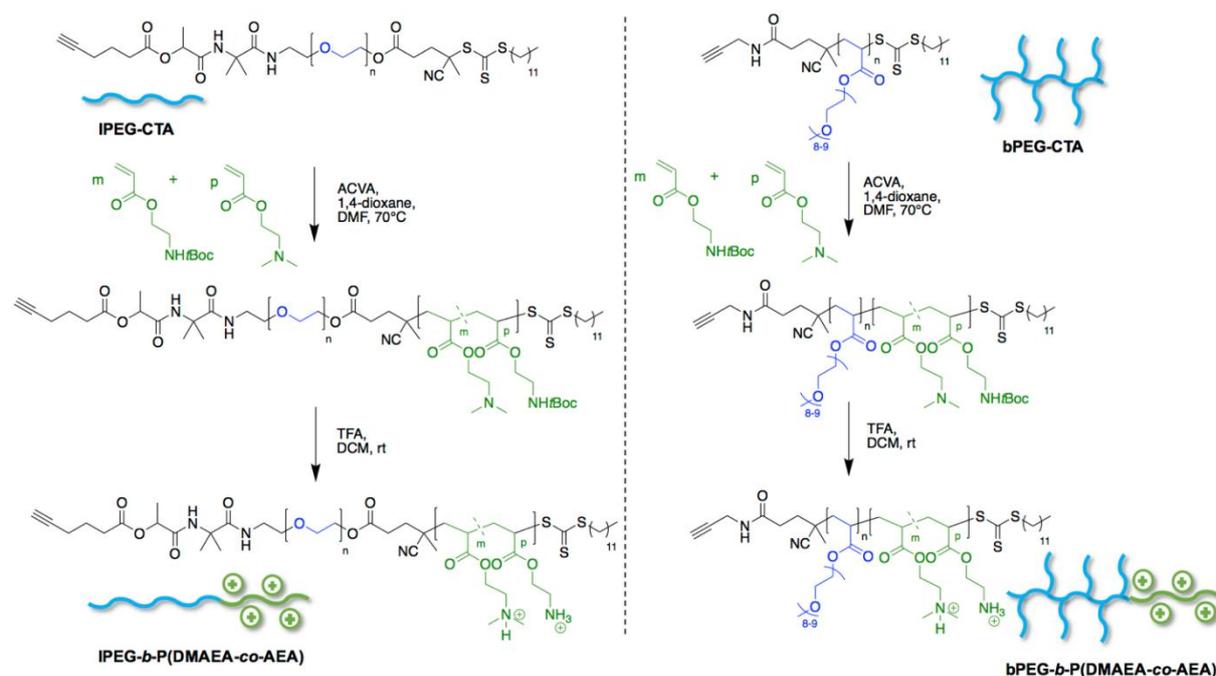
Synthesis of PEGylated, cationic aminoethyl-based polyacrylates

Well-defined PEGylated, cationic polyacrylates containing primary and tertiary ammonium entities with two different PEG topologies (i.e., linear or brush-like) were obtained by RAFT copolymerization of aminoethyl-based acrylates followed by amine deprotection under acidic conditions. As for N-unsubstituted aminoethyl-based polyacrylates, the protection of the primary amine group was necessary to prevent early aminolysis of the chain transfer agent at the chain-end. We have previously reported on the successful synthesis of well-defined N-unsubstituted aminoethyl-based polyacrylates through RAFT polymerization from protected tert-butyloxycarbonylaminoethyl acrylate (tBocAEA) followed by amine deprotection.^{23,24} And we have also established optimized RAFT experimental conditions for the copolymerization of tBocAEA and a N-substituted aminoethyl-based acrylate (N,N-dimethylaminoethyl acrylate (DMAEA)).²⁴ Accordingly, the following experimental conditions were employed: ACVA was used as a conventional radical initiator (ACVA/RAFT agent = 0.2/1) in 1,4-dioxane as the solvent and 70 °C (Scheme 3). Previously synthesized IPEG-CTA and bPEG-CTA were employed as macro-RAFT agents to study the influence of the PEG topology on pDNA complexation and cell viability. Also, the influence of PEGylation was studied by using a non-PEGylated RAFT agent, 2-cyano-5-oxo-5-(prop-2-yn-1-ylamino)pentan-2-yl dodecylcarbonotrithioate (named COPYDC).²⁸ RAFT copolymerizations of DMAEA and tBocAEA with the following initial molar ratio: $[DMAEA]_0/[tBocAEA]_0/[RAFT\ agent]_0/[ACVA]_0 = 50/50/1/0.2$ were launched at 70 °C under argon. Similar overall monomers conversions, as calculated by ¹H NMR spectroscopy (Figures S3, S6 and S9), were obtained in order to target similar molar DMAEA/tBocAEA compositions of (PEGylated) aminoethyl-based polyacrylates (Table 1). The resulting copolymers were purified by six precipitations and analyzed by ¹H NMR spectroscopy (Figures S5, S8 and S11) to determine the molar compositions of the copolymers and by SEC to determine the blocking efficiency. Results reported in Table 1 showed that PDMAEA/PtBocAEA molar compositions were similar for all three copolymers. Reactivity ratios close to 124 ensure an equivalent distribution of the two acrylate-based monomers all along the copolymers chains. The overlaid SEC traces (Figures S4 and S7) of IPEG-CTA, bPEG-CTA and the

resulting copolymers after RAFT copolymerization showed a shift of the SEC trace towards higher molar masses (RI detection). A broadening of SEC chromatograms has been observed for block copolymers. This is due to the adsorption of amino-groups onto the SEC columns as previously reported.³⁵ The combination of both SEC and NMR analyses is however consistent with the formation of IPEG-b-P(DMAEA-co-tBocAEA) and bPEG-b-P(DMAEA-co-tBocAEA) block copolymers.

To synthesize the corresponding cationic copolymers able to complex pDNA, the amine deprotection of the tBocAEA repeating units was considered. tBocAEA units have been deprotected under acidic conditions to form aminoethyl acrylate (AEA) units. Our group showed the efficiency of trifluoroacetic acid (TFA) to deprotect PtBocAEA.^{23,24} Therefore, a similar procedure was used to deprotect the amine groups of tBocAEA repeating units from P(DMAEA-co-tBocAEA), IPEG-b-P(DMAEA-co-tBocAEA) and bPEG-b-P(DMAEA-co-tBocAEA) copolymers. The reactions were performed in DCM with an excess of TFA at room temperature during 5 h (Scheme 3). The final copolymers were purified by several precipitations and analyzed by ¹H NMR spectroscopy. The overlay of ¹H NMR spectra of P(DMAEA-co-tBocAEA) (Figure S12), IPEG-b-P(DMAEA-co-tBocAEA) (Figure S13) and bPEG-b-P(DMAEA-co-tBocAEA) (Figure S14) copolymers before and after amine deprotection showed the disappearance of the signal at 1.37 ppm of the methyl protons C(=O)OC(CH₃)₃ of the tBoc group. Moreover, the shift of the signal of methylene OCH₂CH₂N(CH₃)₂ of DMAEA units, from 2.16 to 3.40 ppm and the shift of the signal of the methyl protons OCH₂CH₂N(CH₃)₂ of DMAEA units, from 2.16 to 2.85 ppm prove the cationization of DMAEA units. NMR analyses are thus relevant showing a complete protonation of both AEA and DMAEA units.

The resulting well-defined non-PEGylated, cationic aminoethyl-based polyacrylates and PEGylated counterparts containing the same molar composition of primary and tertiary ammonium entities were then used to study the impact of PEGylation and PEG topology on pDNA complexation and cell viability.



Scheme 3. Synthesis of PEGylated, cationic aminoethyl-based polyacrylates by successive RAFT copolymerization of DMAEA/tBocAEA and amine deprotection.

Table 1. Data For The RAFT Copolymerizations Of DMAEA And tBocAEA Using COPYDC, IPEG-CTA And bPEG-CTA RAFT agents And ACVA At 70 °C For [DMAEA]₀/[tBocAEA]₀/[RAFT agent]₀/[ACVA]₀ = 50/50/1/0.2.

Expt.	RAFT agent	Conv. ^{a)} (%)	$DP_{n,NMR}$ ^{b)}		$M_{n,NMR}$ ^{c)} (g.mol ⁻¹)	$M_{n,SEC}$ ^{d)} (g.mol ⁻¹)	\bar{D} ^{d)}
			DMAEA	tBocAEA			
1	COPYDC	19	10	12	4 500	5 200	1.50
2	IPEG-CTA	22	11	12	7 500	11 700	1.31
3	bPEG-CTA	20	10	10	9 800	11 200	1.57

a) Total DMAEA and tBocAEA conversion determined by comparing the peak area of the vinylic protons of DMAEA and tBocAEA at 5.79-6.40 ppm with the peak area of the CH of DMF (used as internal standard) at 8.02 ppm. b) Determined by ¹H NMR spectroscopy analyses of purified copolymers, see spectra in Supporting Information (Figures S5, S8 and S11). c) $M_{n,NMR} = M_{n,NMR,RAFT\ agent} (or\ MM_{RAFT\ agent}) + [DP_{n,NMR}(DMAEA) \times MM(DMAEA) + DP_{n,NMR}(tBocAEA) \times MM(tBocAEA)]$ with $M_{n,NMR,IPEG-CTA} = 3440\ g.mol^{-1}$ and $M_{n,NMR,bPEG-CTA} = 6200\ g.mol^{-1}$. d) Determined by polystyrene-calibrated SEC in DMF-LiBr (1 g.L⁻¹) at 60 °C.

pDNA Complexation and cytotoxicity studies

Interaction of negatively-charge pDNA and cationic (PEGylated) aminoethyl-based polyacrylates in aqueous solution resulted in the formation of bioconjugates whose purpose was to compact and protect it from nucleases. Hence, the pDNA complexing ability was investigated by agarose gel electrophoresis (Figure 4) and the impact of PEGylation on the pDNA binding was determined. As shown in Figure 4, all polymers were able to fully complex pDNA and completely retard its mobility. Interestingly, using a cationic, non-PEGylated aminoethyl-based polyacrylate P(DMAEA-co-AEA) led to complete pDNA retardation for N/P = 1. At this ratio, all pDNA was trapped inside the polyplex formed using cationic P(DMAEA-co-AEA) and no residual free pDNA was present. Such a low N/P

ratio is of particular interest as commercially available JetPEI, commonly used for delivery of nucleic acids, a N/P > 2 was needed as recommended by the manufacturer.

To study the impact of PEG topology on the pDNA complexation, the binding ability of IPEG-b-P(DMAEA-co-AEA) and of bPEG-b-P(DMAEA-co-AEA) has been compared. Agarose gel electropherograms showed a complete pDNA retardation for N/P = 3 and for N/P = 6 using linear PEG and brush-like PEG, respectively. Therefore, the efficiency of cationic aminoethyl-based polyacrylates to completely bind pDNA increased as follows: P(DMAEA-co-AEA) < IPEG-b-P(DMAEA-co-AEA) < bPEG-b-P(DMAEA-co-AEA). This suggest that PEGylation, and more specifically a brush-like PEG conformation, together with an increase in PEG length, reduced the accessibility of positive charges of the cationic copolymers due to steric hindrance. However, PEGylation would allow to increase the half-time of polyplexes by reducing interactions with blood components and therefore improve the access to distant tumors. In this context, the cationic IPEG-b-P(DMAEA-co-AEA) copolymer seemed to be a good compromise, zeta-potential and DLS measurements were performed on pDNA/IPEG-b-P(DMAEA-co-AEA) polyplex (N/P = 3). A shift of the zeta potential from positive to neutral value was observed for the polymer after pDNA complexation (from +12 mV to -1 mV), clearly showing the neutralization of the cationic charges of the copolymer and the formation of the polyplexes by electrostatic binding. The hydrodynamic diameter (Dh) of the resulting polyplex was equal to 182 nm (Figure S15) and was in agreement with a compacted pDNA.

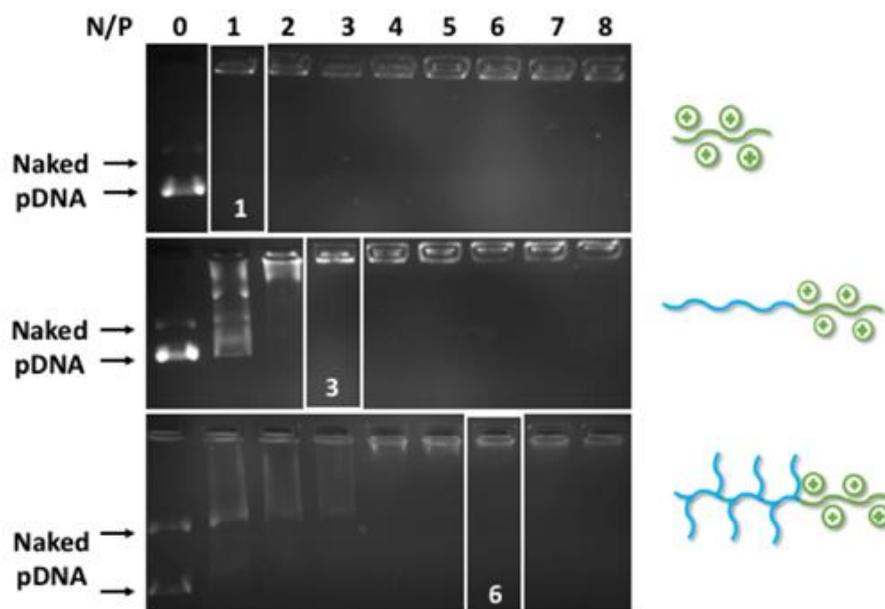


Figure 4. Agarose gel electropherograms of pDNA polyplexes obtained at different N/P ratios using P(DMAEA-co-AEA) copolymer (top), IPEG-b-P(DMAEA-co-AEA) copolymer (middle) and bPEG-b-P(DMAEA-co-AEA) copolymer (bottom).

The potential toxicity of IPEG-b-P(DMAEA-co-AEA), bPEG-b-P(DMAEA-co-AEA) and P(DMAEA-co-AEA) copolymer were tested and compared to the commercial JetPEI on one representative mammalian cell type: murine fibroblast cells (NIH/3T3). The cytotoxicity was evaluated in vitro by MTT assays at three different concentrations (0.5, 1 and 2 mg.mL⁻¹) (Figure 5). PEGylated aminoethyl-based

polyacrylates reduced their cytotoxicity whatever the concentration was, as incubation of P(DMAEA-co-AEA) with NIH/3T3 cells resulted in less than 50% cell viability whereas a higher percentage (> 50%) of viable cells was measured after exposure to PEGylated P(DMAEA-co-AEA) copolymers. These results were in agreement with previously published data⁹ which revealed a reduction of cytotoxicity of PEI upon PEGylation thanks to the shielding of the cationic charges and the consequent reduced interaction with cell membranes. However, these studies only focused on linear PEG.⁹ Herein, we have investigated the role of the PEG topology on the toxicity of PEGylated aminoethyl-based polyacrylates on NIH/3T3 cells (Figure 5). Both IPEG-based and bPEG-based P(DMAEA-co-AEA) copolymers showed low or no cytotoxicity at 0.5 and 1 g.L⁻¹ with more than 80 % of viable NIH/3T3 cells after 72 h incubation. At higher copolymer concentrations, the cell viability of IPEG-based and bPEG-based P(DMAEA-co-AEA) copolymers decreased to 54 % and 77 %, respectively. IPEG-based P(DMAEA-co-AEA) copolymer showed a similar behavior than JetPEI and bPEG-based P(DMAEA-co-AEA) copolymer did not display any toxicity, which is a very encouraging indication of their biocompatibility and their possible safe use as pDNA carriers.

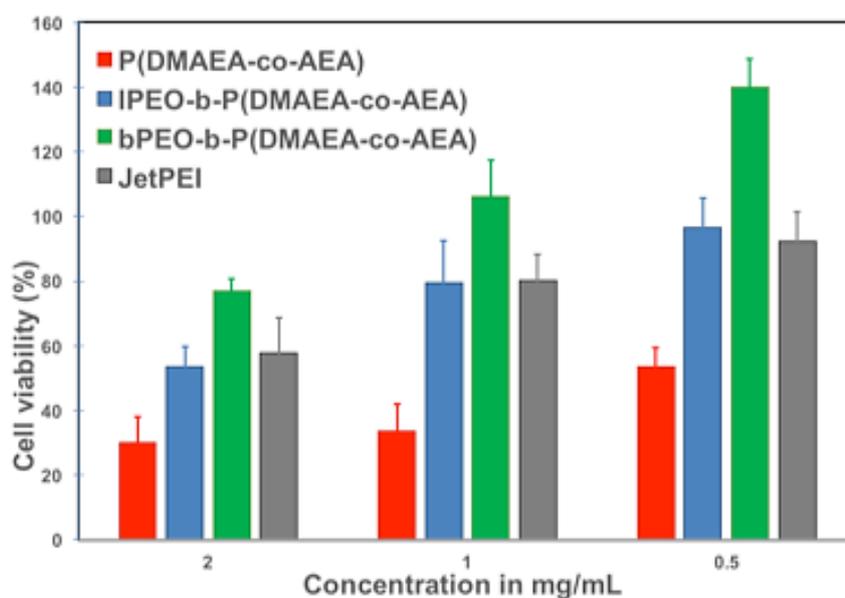


Figure 5. Cell viability (MTT assay) after incubation of NIH-3T3 cells with polyplexes concentrations at 0.5, 1 and 2 mg.mL⁻¹ using P(DMAEA-co-AEA), IPEG-b-P(DMAEA-co-AEA), bPEG-b-P(DMAEA-co-AEA) and JetPEI. Results were expressed as percentages of absorption of treated cells in comparison to that of untreated ones as a control.

Experimental

Materials

All reagents were purchased from Sigma-Aldrich and used as received unless otherwise notified: 4,4'-azobis(4-cyanolaveric acid) (ACVA, Sigma-Aldrich, 98 %), α -amino, ω -hydroxyl-poly(ethylene oxide)

($DP_{n,MALDI} = 64$, Rapp Polymere), plasmid DNA (pDNA, pBR322, $0.5\mu\text{g}\cdot\mu\text{L}^{-1}$, EuroMEDEX), jetPEI (Polyplus-Transfection), Sodium trifluoroacetate (NaTFA, $\geq 98\%$), trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB, $\geq 98\%$), dithranol (DIT, 97%), dicyclohexylcarbodiimide (DCC, 99%), 4-(dimethylamino)pyridine (DMAP, 99%) 4-cyano-4-[(dodecylsulfanylthiocarbonyl)sulfanyl]pentanoic acid (CDP, 97%), hydrochloric acid (HCl, 37%), trifluoroacetic acid (99%), 1,4-dioxane ($\geq 99.8\%$), N,N-dimethylformamide (DMF, $\geq 99.9\%$), hexane ($\geq 95\%$), acetone (pure, Carlo Erba), dimethylsulfoxide (DMSO, Pure, Carlo-Erba), diethyl ether (99.8%, Carlo Erba), methanol (MeOH, 99.8%, Carlo Erba), ethyl acetate (pure, Carlo Erba), phosphorus pentoxide (P_2O_5 , $\geq 99.8\%$), aluminum oxide (basic activated), 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT, $\geq 97.5\%$), ethidium bromide (BioReagent for molecular biology), sodium chloride (NaCl, Acros-Organics, 99.5%), silica gel for chromatography (SiO_2 , 0.035 - 0.070 mm, 60 Å, Acros Organics), Trisacetate EDTA Buffer (TAE 50x, EuroMEDEX), agarose type D-5 (EuroMEDEX), Dulbecco's modified eagle medium (DMEM, Lonza), fetal bovine serum (FBS, Lonza), RPMI 1640 medium (Lonza). 2-(N,N-dimethylaminoethyl) acrylate (DMAEA, Sigma-Aldrich, 98%) was passed through a column of aluminum oxide prior to polymerization. N-(tert-butoxycarbonyl)aminoethyl acrylate (tBocAEA) has been synthesized following a reported procedure.²³ Oligo(ethylene glycol) acrylate (OEGA, pure, Sigma-Aldrich) was passed through a column of aluminum oxide prior to polymerization. α -Alkynyl, ω -hydroxyl-PEG27 and 2-cyano-5-oxo-5-(prop2-yn-1-ylamino)pentan-2-yl dodecylcarbonotrithioate (named COPYDC)²⁸ have been synthesized following reported procedures from our laboratory. Dichloromethane (DCM, HPLC grade, Fisher Chemical) and tetrahydrofuran (THF, 99.9%) were dried over dry solvent stations GT S100. Ultra-pure water was obtained from a reverse osmosis system and had a conductivity of 18.2 M Ω cm at 25°C. All the deuterated solvents are purchased from Euriso-top.

Analytical methods

Nuclear Magnetic Resonance (NMR) spectra were recorded on a Bruker AC-400 spectrometer for ¹H NMR (400 MHz).

The average molar masses (number-average molar mass $M_{n,SEC}$, weight-average molar mass $M_{w,SEC}$ and dispersity ($\mathcal{D} = M_{w,SEC}/M_{n,SEC}$) values were measured by Size Exclusion Chromatography (SEC) using DMF-LiBr (1 g·L⁻¹) as an eluent and carried out using a system equipped with a guard column (Polymer Laboratories, PL Gel 5 μm) followed by two columns (Polymer Laboratories, 2 PL gel 5 μm MIXED-D columns) and with a Waters 410 differential refractometer (RI) and a Waters 481 UV-Visible detector. The RI detector is located before the UV detector in the system, thus explaining the lag between RI and UV traces. The instrument operated at a flow rate of 1.0 mL·min⁻¹ at 60 °C and was calibrated with narrow linear polystyrene (PS) standards ranging in molar mass from 580 g·mol⁻¹ to 483 000 g·mol⁻¹. Molar masses and dispersities were calculated using Waters EMPOWER software.

MALDI-TOF Mass Spectrometry analyses were performed on a Bruker UltraFlex II instrument equipped with a nitrogen laser operating a 337 nm, pulsed ion extraction source and reflectron. Spectra were recorded in the reflectron mode with an acceleration voltage of 25 kV and a delay of 300 ns. 500 single shot acquisitions were summed to give the spectra. Samples were prepared by dissolving the matrix, the DCM and mixing with the polymer. NaTFA in acetone had been deposited to act as a cationizing agent.

Zeta potential (ξ) and size measurements were performed using a dynamic light scattering (DLS) instrument (Zetasizer Nano ZS, Malvern Instrument) equipped with a He-Ne laser beam at 658 nm. Average values were obtained from four replicate measurements.

Polymer syntheses

Synthesis and characterization of the linear PEG macromolecular RAFT agent (IPEG-CTA)

In a round bottom flask were charged a magnetic stir bar, α -alkynyl, ω -hydroxyl-PEG (2.19 g, 0.674 mmol), DMAP (17.6 mg, 0.144 mmol), and 4-cyano-4-[[dodecylsulfanylthiocarbonyl]sulfanyl]pentanoic acid (CDP, 543.1 mg, 1.34 mmol). 10 mL of anhydrous DCM were then added under argon. In another round bottom flask, DCC (302.4 mg, 1.47 mmol) was solubilized in 7 mL of anhydrous CH_2Cl_2 under argon. The solution of DCC was injected dropwise into the first solution via a syringe and the resulting mixture became trouble. It was further degassed for 10 min and the reaction was launched at room temperature for 4 days. The organic phase was washed twice with a solution of HCl 3 M and twice with a saturated solution of NaCl. The organic phase was evaporated and the residual mixture was kept in ice. Aliquots of 500 mg of the crude product were purified using a silica column (15 mL of silica) with DCM/MeOH mixture (from 100/0 to 90/10 v/v). The solvent was evaporated and the product was dried under vacuum at 40 °C and further analyzed by MALDI-TOF mass spectrometry (Figure 1) and ^1H NMR spectroscopy (Figure S1). $M_{n,\text{NMR}} = 3440 \text{ g}\cdot\text{mol}^{-1}$. SEC in DMF/LiBr: $M_{n,\text{SEC}} = 8200 \text{ g}\cdot\text{mol}^{-1}$ (PS equivalent), $\mathcal{D} = 1.13$.

Synthesis and characterization of the brush-like PEG macromolecular RAFT agent (bPEG-CTA)

In a Schlenk flask were charged a magnetic stir bar, ACVA (8.5 mg, 0.030 mmol), COPYDC (134.2 mg, 0.305 mmol) and OEGA (4.3275 g, 9.02 mmol) in 4 mL of DMF. The solution was homogenized and degassed with argon for 30 min. The reaction was performed at 70 °C. For kinetic study, during RAFT polymerization, aliquots were withdrawn to determine OEGA conversion by ^1H NMR spectroscopy, $M_{n,\text{SEC}}$ and \mathcal{D} values by SEC in DMF (LiBr 1 $\text{g}\cdot\text{L}^{-1}$). For a high amount of bPEG-CTA, the reaction was stopped by freezing the Schlenk in liquid nitrogen and opening it to the air after 135 min. OEGA conversion was determined to be 52 % by ^1H NMR spectroscopy by comparing the integration area values of the formamide proton of DMF at 8.00 ppm and the vinylic protons of OEGA between 5.7 and 6.5 ppm. The crude polymer was purified by several dialysis in pure water, lyophilized and dried under vacuum in the presence of P_2O_5 . The pure product was analyzed by ^1H NMR spectroscopy (Figure S2) $M_{n,\text{NMR}} = 6200 \text{ g}\cdot\text{mol}^{-1}$ and SEC in DMF (LiBr 1 $\text{g}\cdot\text{L}^{-1}$): $M_{n,\text{SEC}} = 14700 \text{ g}\cdot\text{mol}^{-1}$, $\mathcal{D} = 1.26$.

RAFT copolymerizations of DMAEA and tBocAEA

Using a linear PEG macromolecular chain transfer agent for IPEG-b-P(DMAEA-co-tBocAEA) copolymers. In a typical RAFT copolymerization procedure, a magnetic stir bar was charged to a round bottom flask together with tBocAEA (0.417 g, 1.93×10^{-3} mol), DMAEA (0.276 g, 1.93×10^{-3} mol), IPEG-CTA (0.135 g, 3.86×10^{-5} mol), ACVA (2 mg, 7.71×10^{-6} mol), 1,4-dioxane (4.1 mL) and DMF (0.07 mL) used as the internal reference. The mixture was deoxygenated by bubbling argon for 30 min. The solution was then immersed in an oil bath thermostated at 70 °C to allow the polymerization to occur. The reaction was stopped after 5h30 by opening the reaction mixture to oxygen. Overall monomers conversion (DMAEA and tBocAEA) was determined to be 22% by ^1H NMR spectroscopy (Figure S3). 1,4-Dioxane and DMF were removed under vacuum. The resulting product was then dissolved in DCM, precipitated in cold hexane/diethyl ether (90/10 v/v), filtered and dried under vacuum. The

final product, obtained as a yellow solid, was characterized by SEC analysis and ¹H NMR spectroscopy (Figures S4 and S5, respectively).

Using a brush-like PEG macromolecular chain transfer agent for bPEG-b-P(DMAEA-co-tBocAEA) copolymers. In a typical RAFT copolymerization procedure, a magnetic stir bar was charged to a round bottom flask together with tBocAEA (0.396 g, 1.84×10^{-3} mol), DMAEA (0.271 g, 1.89×10^{-3} mol), bPEG-CTA (0.334 g, 3.68×10^{-5} mol), ACVA (2 mg, 7.32×10^{-6} mol), 1,4-dioxane (10 mL) and DMF (0.3 mL) used as the internal reference. The mixture was deoxygenated by bubbling argon for 30 min. The solution was then immersed in an oil bath thermostated at 70 °C to allow the polymerization to occur. The reaction was stopped after 27 h by opening the reaction mixture to air. The overall monomers conversion (DMAEA and tBocAEA) was determined to be 20% by ¹H NMR spectroscopy (Figure S6). 1,4-dioxane and DMF were removed under vacuum. The resulting product was then dissolve in DCM, precipitated six times in cold hexane/diethyl ether (90/10 v/v), centrifuged (7000 rpm, 5 min, 0 °C) and dried under vacuum. The final product obtained as a yellow solid was characterized by SEC analysis and ¹H NMR spectroscopy (Figures S7 and S8, respectively).

Using COPYDC as a chain transfer agent for P(DMAEA-co-tBocAEA) copolymers. In a typical RAFT copolymerization procedure, a magnetic stir bar was charged to a round bottom flask together with tBocAEA (3.612 g, 1.68×10^{-2} mol), DMAEA (2.160 g, 1.51×10^{-2} mol), COPYDC (0.135 g, 3.07×10^{-4} mol), ACVA (8 mg, 3.00×10^{-5} mol), DMF (6.3 mL). The mixture was deoxygenated by bubbling argon for 30 min. The solution was then immersed in an oil bath thermostated at 70 °C to allow the polymerization to occur. The reaction was stopped after 21 h by opening the reaction mixture to air. Total monomers conversion of DMAEA and tBocAEA was determined to be 19% by ¹H NMR spectroscopy (Figure S9). DMF was removed under vacuum and the resulting product was dissolve in acetone, precipitated six times in cold hexane/diethyl ether (90/10 v/v), centrifugated (7000 rpm, 5 min, 0 °C) and dried under vacuum. The final product obtained as a yellow oil was characterized by SEC analysis and ¹H NMR spectroscopy (Figures S10 and S11, respectively).

Synthesis and characterization of cationic ((I or b)PEG-b-)P(DMAEA-co-AEA)copolymers

In a typical procedure, 100 mg of IPEG-b-P(DMAEA-co-tBocAEA) (13.9×10^{-5} mol) block copolymer was dissolved in 2 mL of DCM. TFA (214 μL, 2.78×10^{-3} mol) was added dropwise at 0 °C. After completed addition, the solution was stirred during 5h at room temperature. DCM was then removed using a rotary evaporator and the polymer was dissolved in acetone, precipitated into diethyl ether/hexane (50/50 v/v), centrifuged (7000 rpm, 0 °C, 5 min) and dried under vacuum. The product obtained was a yellow solid and analyzed by ¹H NMR spectroscopy (Figure S12 for P(DMAEA-co-AEA), Figure S13 for IPEG-b-P(DMAEA-co-AEA) and Figure S14 for bPEG-b-P(DMAEA-co-AEA)).

Gel retardation assay for polymer/pDNA polyplexes

Cationic copolymers/pDNA polyplexes were prepared at various N/P ratios by mixing pDNA (pBR322, $0.5 \mu\text{g}\cdot\text{L}^{-1}$) with a solution of copolymers in tris-HCl solution at pH = 7.4 (nitrogen concentration = $0.5 \mu\text{g}\cdot\mu\text{L}^{-1}$), DNA loading 6X and completed with the pH buffering solution. Quantities are detailed in Table S1. The solution was homogenized with a Vortex-2 (Genie scientific industries) during 10 min. The gel was prepared using 0.8 % of agarose in TAE 1 X buffer (Tris, Acetate, EDTA) containing ethidium bromide. The gel retardation assay was carried out at 50 mV for 45 min. with TAE 1 X running buffer. Naked pDNA was used as control. The migration of pDNA was observed with a UV illuminator (Fisher BioBlock Scientific) and images were acquired using Vision-Capt software.

Cationic copolymers/pDNA polyplexes were prepared at various N/P ratios by mixing pDNA (pBR322, $0.5 \mu\text{g}\cdot\text{L}^{-1}$) with a solution of copolymers in tris-HCl solution at pH = 7.4 (nitrogen concentration = $0.5 \mu\text{g}\cdot\mu\text{L}^{-1}$), DNA loading 6X and completed with the pH buffering solution. Quantities are detailed in Table S1. The solution was homogenized with a Vortex-2 Genie scientific industries during 10 min. The gel agarose was prepared using 0.8 % of agarose in TAE 1 X buffer (Tris, Acetate, EDTA) containing ethidium bromide. The gel retardation assay was carried out at 50 mV with running TAE 1 X buffer. Naked pDNA was used as control. The migration of pDNA was observed with a UV illuminator (Fisher BioBlock Scientific) and photos were taken.

Cytotoxicity studies

Cell culture conditions. The embryonic murine fibroblast (NIH-3T3) obtained from ATCC (reference CRL-1658™) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Belgium), supplemented with $50 \text{ U}\cdot\text{mL}^{-1}$ penicillin, $50 \text{ U}\cdot\text{mL}^{-1}$ streptomycin and 10% fetal bovine serum (FBS, Lonza, Belgium). Cell were maintained at 37°C in a humidified 5% CO_2 atmosphere.

MTT test. The in vitro cytotoxic activity of the different copolymers was evaluated using the MTT test. Cells were seeded in $100 \mu\text{L}$ of growth medium ($4 \times 10^4 \text{ cells}\cdot\text{mL}^{-1}$) in 96-well plates (TPP) and allowed to adhere for 24 h. Initial cell density and incubation time were determined to allow cells to remain in exponential growth and to undergo two cell-doubling times during the assay. Cells were then exposed to the polymers, opportunely diluted in complete cell medium, for 48 h.

At the end of the incubation period, $20 \mu\text{L}$ of a $5 \text{ mg}\cdot\text{mL}^{-1}$ MTT solution in phosphate buffered saline was added to each well. After incubation, the culture medium was removed and replaced by $200 \mu\text{L}$ of DMSO to solubilize the formazan crystals and obtain a purple solution. The absorbance of the solubilized dye was measured spectrophotometrically with a microplate reader (LAB System Original Multiscan MS) at 570 nm. The percentage of viable cells for each treatment was calculated from the ratio of the absorbance of the well containing the treated cells versus the average absorbance of the control wells (i.e., untreated cells). All experiments were repeated three times to determine means cell-viability and standard-deviations.

Conclusions

Aminoethyl-based polyacrylates linked either to a linear PEG or a brush-like PEG have been successfully synthesized by successive RAFT polymerization and amine deprotection. These copolymers allowed the effect of PEGylation and PEG topology on pDNA complexation and cytotoxicity to be finely investigated. Among all copolymers architectures, keeping constant the primary ammonium and tertiary ammonium molar content, brush-like, PEGylated aminoethyl-based polyacrylates induced the lowest cytotoxicity and the highest N/P ratio to fully complex pDNA whereas the linear PEGylated ones fully complexed pDNA with a low N/P ratio (= 3) and showed the absence of cytotoxicity against NIH/3T3 cell lines even at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$. It has to be noted that the alkyne end-chain of cationic linear and brush-like PEGylated aminoethyl-based polyacrylates (Scheme 1) offers the opportunity for further modification of the polymers, thus enlarging the field of application of these polymers in bioconjugation.

Conflicts of interest

There are no conflicts to declare.

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