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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.

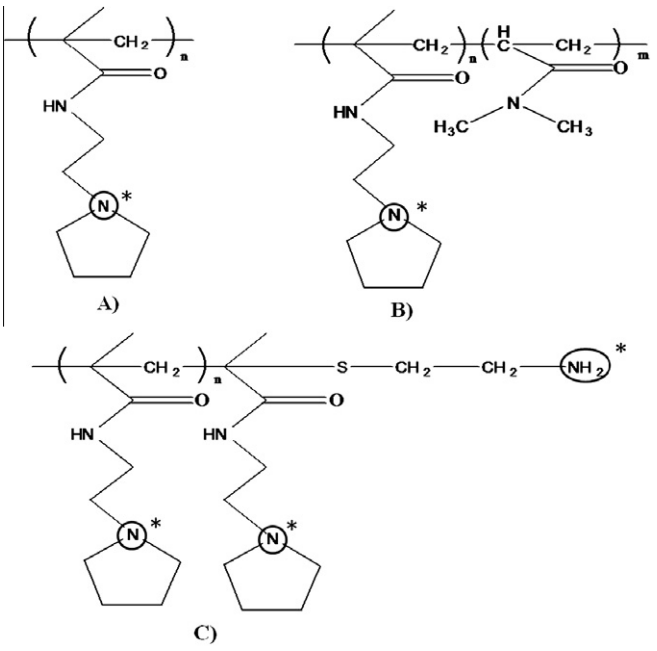
2.3. Characterization of the polymer systems

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.

2.3.3. Thermal analysis

The glass transition temperatures, Tg, of the polymers were determined by Differential Scanning Calorimetry, DSC, using a Perkin–Elmer DSC-7 calorimeter. Typical sample weights were 6–8 mg. Thermal transition temperature measurements were conducted by heating the samples from 0 to 200 °C at 10 °C min^{-1} taking as Tg the onset point.

2.3.4. Determination of dissociation constants (pK_a)

The pK_a of the polymers were determined by acid–base titration of a 100 mg of monomer, homopolymer or telomer solution in a 25 mL aqueous 0.1 M NaCl. Diluted aqueous solutions of NaOH were used to complete the titration using small volumes in order to avoid modifying the ionic strength. One to three millilitre of a 0.1 N HCl solution was added to ensure the ionization of the amine groups of the telomers. The changes of pH were measured with a Schott GC841 pH metre.

2.3.5. Determination of free —NH_2 groups by ninhydrin assay

Two milligram of each oligomer was dissolved in separate 1.5 mL microcentrifuge tubes. To each tube was added 200 μL of deionized water and 1 mL of ninhydrin solution (one part of 4% (w/v) ninhydrin in 2-ethoxyethanol and one part 200 mM citric acid with 0.16% (w/v) stannous chloride, pH 5.0). The tubes were heated at 95 °C for 30 min on a heating block. A deep blue or purple colour chromophore (Ruhemann's purple) was obtained. The tubes were cooled to room temperature, and 250 μL of the cooled solution was added to 1 mL of 50% (v/v) isopropanol solution in water. This mixture was vortexed, and the optical absorbance of the solution was recorded with a spectrophotometer (UV 1601 Shimadzu Europe Ltd., Duisburg, Germany) at a wavelength of 570 nm.

2.4. Polyplex formation and characterization

Various formulations of polymer/DNA (1 μg of DNA) complexes in a phosphate buffered saline (PBS, pH 7.4) were prepared with phosphate/nitrogen (P/N) ratios ranging from 2 to 20 with gentle vortexing. The complexes were analysed after 1 h incubation at room temperature, and the complexes were then used for analysis.

2.4.1. Agarose gel electrophoresis

For the electrophoresis experiment, a 0.9% (w/v) agarose gel was used and the migration was carried out under 100 V for 1 h. Naked DNA (1 μg) was used as a marker. After electrophoresis, DNA bands were visualized by exposure to UV light (G-BOX Chemi XL, United Kingdom).

2.4.2. Determination of average particle size and zeta potential

The determination of particle size of polycation/DNA (1 μg of DNA) complexes was carried out at 25 °C using a size analyser (N5 Submicron Particle Size Analyzer, 2003 Beckman Coulter, Inc. Miami, FL 33196) and Dynamic light scattering (DLS) (Nano-ZS, Malvern instrument). The surface charge of the polymer/DNA (2 μg of DNA) complex was assessed using a Zetamaster system (Malvern Instruments). Every measurement was carried out in three serial measurements. The data acquisition was done with the ALV-Correlator Control Software, and the counting time varied for each sample from 300 s up to 600 s.

2.4.3. Morphological analysis by TEM

TEM measurements were performed using a Hitachi H-7500 microscope. The TEM samples were prepared by depositing the different telomer/DNA (2 μg of DNA) solutions on a carbon-coated copper grid, followed by air-drying.

2.5. In vitro toxicity assay

2.5.1. Cell seeding

For this study, rat 3T3 fibroblasts were used. Cells were routinely grown in Dubecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, Ireland) supplemented with 10% foetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S). Medium was changed every 2–3 days. Cells were sub-cultured weekly using trypsin/EDTA and maintained at 37 °C in a humidified atmosphere of 5% CO_2. After 10 passages, 20,000 cells per cm^2 were seeded. After 24 h seeding in a 96-well plate, medium was changed for a minimum medium (DMEM non-supplemented). All the systems from 1/2 to 1/20 (P/N) ratios with DNA (1 μg) and without DNA, incubated beforehand for 1 h at room temperature for complexation, were added to each well for transfection. After 4 h, medium was changed for complete medium. Cells were cultured for 4 days at 37 °C in a humidified atmosphere of 5% CO_2. Medium was changed every 2–3 days. Cells with no treatment and naked DNA were used as control.

2.5.2. Cell viability

Cell viability was measured using the AlamarBlue™ cell metabolic assay. This assay was performed after 2 and 4 days. Cells were washed with Hank's Balanced Salt Solution (HBSS). AlamarBlue™ (BioSource® International, Invitrogen, Ireland), diluted by a factor of 10 in HBSS, was added to each well. After 3 h of incubation, the absorbance of each sample was measured in a 96-well plate, at wavelengths of 550 and 595 nm, using a microplate reader (VICTOR3 V™ Multilabel Counter, PerkinElmer BioSignal Inc., USA). The percentage of AlamarBlue™ reduction was calculated using a correlation factor R_O in accordance with the supplier's instructions.

2.5.3. Cell proliferation

After culture, 250 μL of water was added in each well. Then, samples were frozen and thawed three times. Picogreen® assay (Invitrogen, Ireland) was used according to the instructions provided by the supplier. The DNA content of the samples was quantified by interpolating values from a linear standard curve.

2.6. Transfection studies

Transfection experiments were performed with 3T3 fibroblasts using the plasmid Gaussian luciferase as reporter gene. Cells were routinely grown in DMEM supplemented with 10% FBS and 1% P/S. Prior to the addition of complexes, 20,000 cells per cm^2 were seeded 24 h on a 48-well plate. Different polymer/plasmid DNA (1 μg) molar ratios ranging from 1/2/ to 1/20 (P/N) were used to prepare the polyplexes. A poly (lysine)/DNA and poly (dimethylaminoethyl methacrylate)/DNA formulations prepared at different polymer/DNA ratios were used as control. The incubation of the polyplexes with the cells was performed either in the presence or absence of serum. In a standard transfection experiment, cells were incubated with desired amounts of polyplexes (200 μL dispersion with 1 μg plasmid DNA per well) for 4 h in a humidified 5% CO_2 atmosphere. Then, medium was removed, and fresh culture medium was added. Cells were cultured for 2 and 4 days. Medium was collected and analysed after each time point for the production of luciferase protein. The assay was performed using the Gaussian princeps luciferase assay kit (New England Biolabs) according to supplier's instructions. The luciferase expression was quantified by measuring luminescence with a plate reader (VarioSkan). All transfection assays were carried out in triplicate.

2.7. Blood compatibility

Human blood was drawn from healthy volunteers into vacutainers containing either EDTA or sodium citrate. All four different sizes and modified surfaces were tested to elucidate the effect of size and charge of oligomers on various components of blood and to determine their effect on erythrocytes, coagulation and the complement system. Ethical approval was granted by the Human Ethics Committee of the National University of Ireland, Galway.

2.7.1. Haemolysis

EDTA-anti-coagulated blood was centrifuged for 5 min at a speed of 900 g. The serum fraction was removed, and the volume was raised to its original using 150 mM NaCl. This step was repeated twice, and the final suspension was diluted 1:10 with 100 mM phosphate buffer. 2×10^8 red blood cells/mL were incubated with the telomers each at a final concentration of 100 μg/mL. Treatment with PBS was used as a negative control, and Triton X-100 1% (w/v) was used as a positive control. All samples were incubated under gentle agitation for 2 h at 37 °C and centrifuged at 900 g for 5 min. The absorbance of the supernatant was measured for release of haemoglobin at 545 nm.

The percentage of haemolysis was calculated as follows:

$$\text{Haemolysis } (\%) = \frac{(\text{Aa} - \text{Ac}) \times 100}{\text{Apc}} \quad (1)$$

where Aa, Ac and Apc are the absorbance of test sample, absorbance of control and highest absorbance for positive control, respectively.

2.8. Statistical analysis of the data

One-way ANOVA was performed using Statistical 6.0 software (Statsoft, Tulsa, USA). Results were considered as significantly different at $p < 0.05$.

3. Results and discussion

3.1. Synthesis and characterization of linear polymers and oligomers

In addition to the synthesis of the homopolymer EPA, two copolymers of EPA/DMA were prepared with the aim of decreasing the excess of positive charge side chain (the tertiary amine) and to increase the hydrophilic nature of the EPA polymer. The EPA and DMA feed molar fractions were 0.68 and 0.32 (corresponding to EPA and DMA weight percentage of 80% and 20%, respectively) and 0.45 and 0.55 (corresponding to EPA and DMA weight percentage of 60% and 40%, respectively). The signals used for the determination of composition of the copolymers (Fig. 1) are those that appear at 3.3 ppm to the dimethyl CH_3—N—CH_3 protons of DMA that increase in intensity with the increase in the molar fraction from 0.32 to 0.55 and those at 3.7 ppm to the NH—CH_2— protons of EPA that decrease in intensity with the decrease in the molar fraction from 0.68 to 0.45.

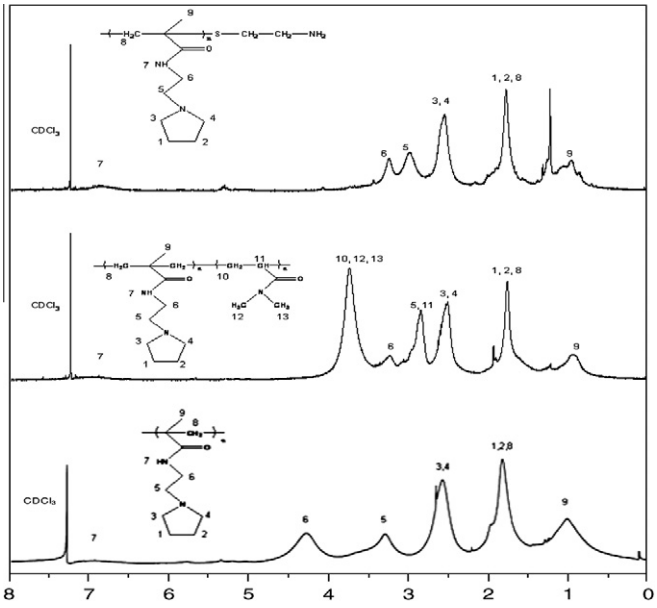


Fig. 1. ^{1}H NMR spectra of the poly-EPA homopolymer, poly (EPA-co-DMA) copolymer and poly-EPA-NH_2 oligomer.

A series of three oligomers at different concentrations of cysteamine (0.03, 0.015 and 0.0075) also were synthesized and labelled as CTA/EPA 3, CTA/EPA 1.5 and CTA/EPA 0.75, respectively. The yield of the DMA and EPA copolymerizations after 24 h of reaction was about 80–85%, whereas the telomerization reactions were 60%.

^{1}H NMR ($CDCl_3$) (ppm) and FTIR signal assignments, stretching vibrations, υ (cm^{-1}) (see also Fig. 1).

Poly-EPA. IR: υ (NH) 3346, υ (C—H) 2964–2814, υ (C=O) 1630, υ (N—CH_2) 1203.

Poly (DMA-co-EPA). IR: υ (NH) 3546 (EPA), υ (C—H) 2964–2814 (EPA, DMA), υ (C=O) 1625 (EPA, DMA), υ (—CH_3,) 1460 (EPA, DMA), υ (N—C) 1330 (EPA, DMA), υ (N—CH_2) 1203 (EPA), υ (—CH_2—) 750 (EPA, DMA).

Poly-EPA/CTA. IR: No significant differences were seen between the different telomers and the homopolymer.

Table 1 shows a summary of the polymer system characterization. It can be noted that the high molecular weight species (120–80 kDa) are the poly-EPA and the EPA–DMA copolymers whereas oligomers (CTA/EPA) have lower molecular weight (2.6–8 kDa) and lower polydispersity.

Telomerization is a process of free radical polymerization in the presence of a given amount of a strong transfer agent (in this case cysteamine). The result is the inactivation of a growing polymer chain by the addition of the transfer molecule, controlling the molecular weight of the polymer and giving chain end with the structure and functionality of the transfer agent.

Table 1
Characterization of the homopolymer, copolymers and oligomers: molecular weight, polydispersity index, ninhydrin assay, glass temperature and dissociation constants.

Sample	Mw [SEC] (kDa)	Polydispersity index	Ninhydrin [NH_2] (μmoles/mL)	DSC Tg (°C)	Titration pK_a
Poly-EPA	120	2.5	–	141	5.6
EPA/DMA 80/20	80	4.1	–	137	5.8
EPA/DMA 60/40	78	4.5	–	126	5.8
CTA/EPA 3 oligomer	2.6	1.3	3.7×10^{-3}	129	5.6 and 9.8
CTA/EPA 1.5 oligomer	3.8	1.5	1.3×10^{-3}	145	5.6 and 9.8
CTA/EPA 0.75 oligomer	8	1.9	0.6×10^{-3}	152	5.6 and 9.8

The ninhydrin assay was used to quantitatively assess the amount of primary amines present in the oligomers. Among the oligomers, the primary amine content increased with increasing cysteamine feed concentration, indicating an increasing incorporation of primary amine groups in the structure as it can be seen with CTA/EPA 3 telomer compared with CTA/EPA 1.5 and CTA/EPA 0.75 [30].

The Tgs of the corresponding oligomers, higher than 100 °C in all cases, were found to be between 126 and 152 °C. The Tg of the homopolymer poly-EPA was 141 °C, higher than the copolymers (Tg of the DMA homopolymer: 89 °C). The Tg of the oligomers depends on the amount of cysteamine, and the variation can lead to the formation of H-bonding interactions between the chain ends —NH_2 groups and the carbonyls in the polymer.

The ionizable character of the polymers was studied by the determination of the dissociation constants pK_a. The presence of tertiary amine side groups in the prepared systems confers the partial or total ionization of the polymers with pK_a values between 5.6 and 5.8. The presence of primary amine groups at the end of the chains of the telomers confers an extra ionization at 9.8, which can play an important role in the transfection process by its properties of condensation with the DNA and buffering capacities inside the cell.

3.2. Characterization of DNA polyplexes

Agarose gel electrophoresis (Fig. 2) showed the capability of the homopolymer to bind DNA. However, the efficiency of binding is not total as observed by the electrophoretic mobility of DNA condensed with the polymer at P/N ratio of 1:10. This mobility can be attributed to the lack of total ionization of the homopolymer at physiological pH due to its pK_a (5.6). More homopolymer is required to complex with DNA. In contrast, the copolymers and telomers were capable of binding DNA in all ratios and from P/N of 1/2 to 1/20, respectively. In case of the copolymers, apart from ionic interactions, the complexation is due to the existence of other interactions with the DNA such as hydrogen bond, Van der Waals interactions and an increase in the pK_a (5.8) increasing the number of protonated amines at physiological pH. The complexation in all the ratios can be attributed to the extra ionization of the primary amine groups (pK_a = 9.8) in the oligomers.

The particle size measurements showed a strong dependence on size and aggregation with the formulation P/N ratio, as is generally described in the literature [31,16]. For all the P/N ratios, the copolymers were able to condense DNA into polyplexes of a mean average size in the range 300–450 nm (Fig. 3a). Their size was higher compared to that of the telomers because of the possible aggregation between the complexes due to a decrease in the charge in its structure and the presence of non-protonated amines in the polymer backbone (pK_a 5.8). In terms of oligomer chemical structure impact on teloplex size and stability (with respect to aggregation), the behaviour of telomers as compared with copolymers was noteworthy. The lower the polymer system molecular weight was, the smaller the polyplexes size was. The size of the teloplexes was between 50 and 250 nm, increasing the size of the teloplexes with the increase in the P/N ratios. It was observed with copolymers that, when the amount of DNA was increased (50 μg), the polydisperse population of large-sized polyplexes further precipitated. Stable formulations were observed only with the oligomers.

The autocorrelation functions of the CTA/EPA 1.5 polyplexes at different (P/N) ratios are presented in Fig. 3b). It can be observed that no differences were found when the polyplexes were dissolved in PBS or medium. The addition of medium + serum to the polyplexes leads to a shift in the autocorrelation towards higher relaxation times, indicating a greater increase in the aggregate size and therefore to the existence of interactions between the excess of positive charge of the polyplexes and the proteins present in the serum.

Measurement of ζ-potential (Fig. 4) also showed that the copolymers and oligomers were capable of binding DNA, with all ζ-potentials measured in the range 16–37 mV, compared with the ζ-potential of free DNA (−31.1 mV, approximately). One aspect to consider is the decrease in the ζ-potential of the polyplexes of the copolymers compared with the oligomers. This is due to the distribution of the ionizable groups in the copolymers and telomers. In case of the copolymer chains, the ionizable groups are well separated by sequences of DMA units (very hydrophilic entities)

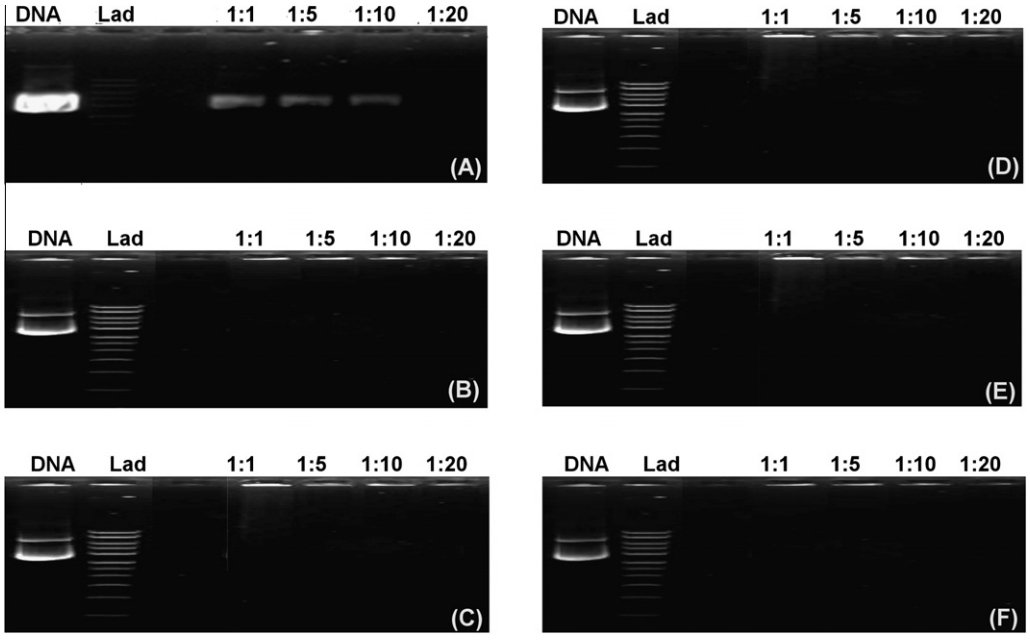


Fig. 2. Electrophoretic mobility of plasmid DNA in polymer/DNA polyplexes with different P/N ratios. (A) EPA polyplexes. (B) 80 EPA/20 DMA polyplexes. (C) 60 EPA/40 DMA polyplexes. (D) CTA/EPA 3 polyplexes. (E) CTA/EPA 1.5 polyplexes. (F) CTA/EPA 0.75 polyplexes.

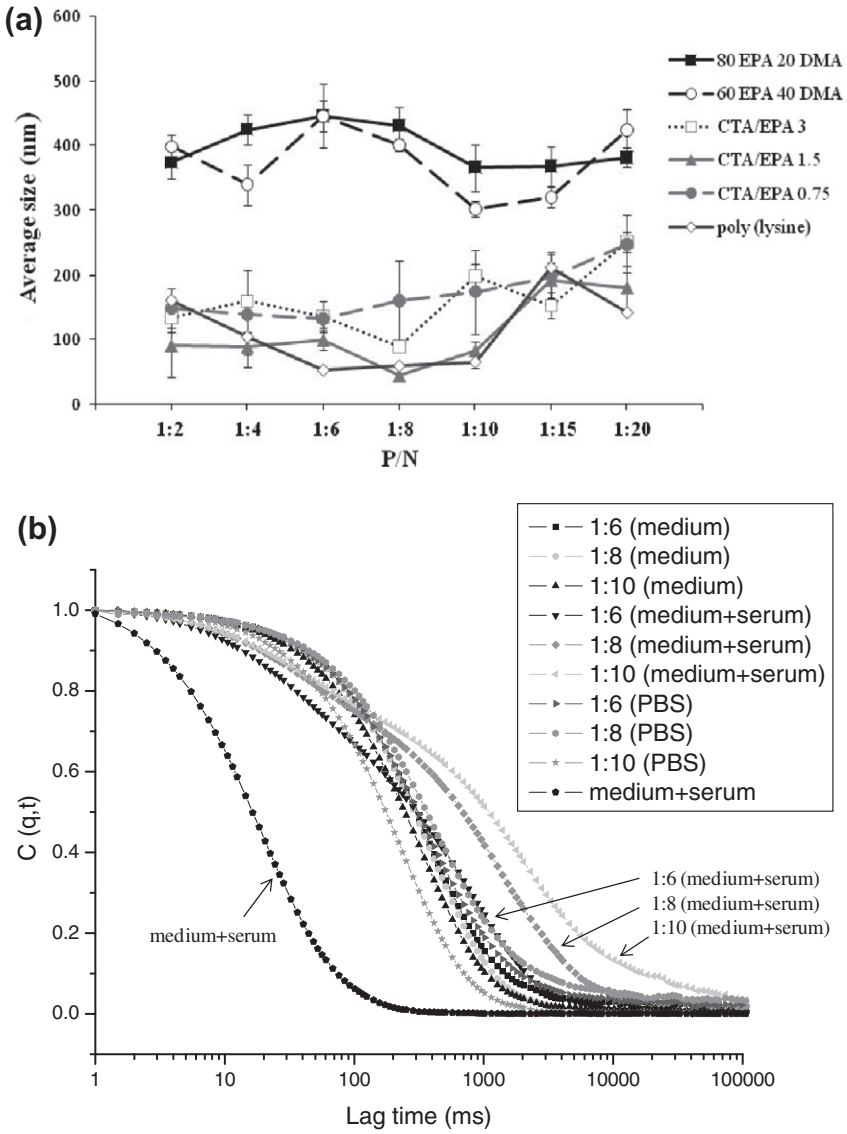


Fig. 3. (a) Average size of various polymer/DNA polyplexes measured at different P/N ratios. (b) Autocorrelation functions of CTA/EPA 1.5 polyplexes at 1:6, 1:8, 1:10 P/N ratios in the presence of medium, medium + serum and PBS.

and therefore the charges are homogeneously distributed in all the nanoparticle volume. However, in case of oligomers, the charges are more concentrated and oriented towards the more hydrophilic medium that is the surface of the nanoparticle dispersed throughout the cell medium. The ζ-potential of the polyplexes of the copolymers was very similar and lower than 20 mV. This suggests that, at these mixing ratios, interaction between the complexes could easily occur to their weak surface charge (small absolute zeta potential values compared to the teloplexes) resulting in the formation of larger complexes [32]. In case of the telomers, the ζ-potential was higher than 20 mV, reaching 36 mV in some cases. These results indicate that oligomers were more charged than were the polymers due to the presence of primary amine, which conferred on them an extra positive charge.

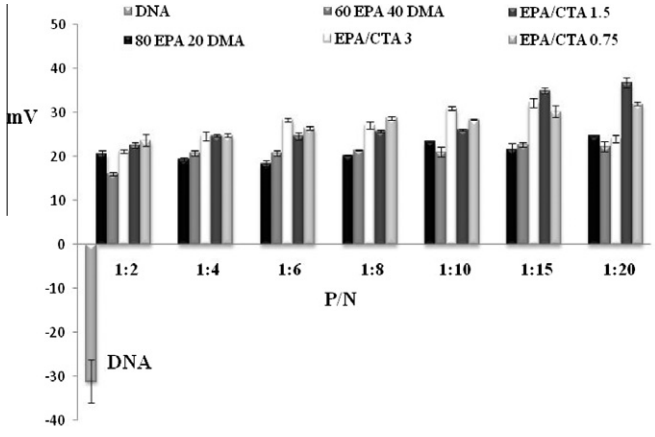


Fig. 4. Zeta potential of various polymer/DNA polyplexes measured at different P/N ratios.

TEM micrographs of the polyplexes showed different shapes depending on the polymer and concentration. In case of the copolymers, the shape observed was spherical for 80EPA/20DMA P/N 1/2 with diameters of between 350 and 450 nm (Fig. 5A). When the P/N ratio increases to a value of 1/6, it is possible to observe other different morphology as a consequence of the relative aggregation of the polyplexes giving nanorods, apparently (Fig. 5B). This situation is also observed for the complexes obtained with poly-L-lysine (Fig. 5C). The polyplexes of the telomers can be observed with a spherical or elliptical shape and a diameter of between 120 and 190 nm (Fig. 5D), where no differences appeared

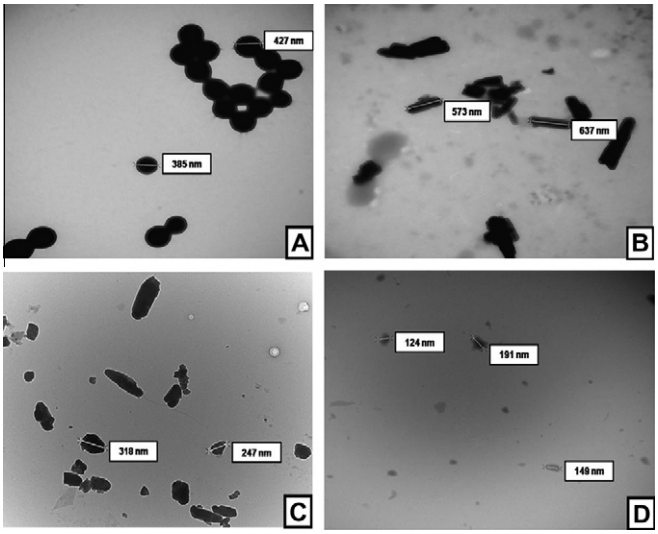


Fig. 5. TEM micrographs of the: (A) 80 EPA/20 DMA 1:2 P/N ratio, (B) 80 EPA/20 DMA 1:6 P/N ratio, (C) poly-L-lysine 1:6 P/N ratio, (D) CTA/EPA3 1:2 P/N ratio polyplexes.

when the P/N ratio increased, indicating that in this case the ratio P/N does not affect specifically the shape and size of the aggregates.

3.3. Cytotoxicity of polymer systems

Cell metabolic activity and cell proliferation after treatment by all systems tested were evaluated by AlamarBlue™ (AB) and Picogreen® assays, respectively. The Fig. 6a) presents the metabolic activity for all the polyplexes and teloplexes with the formulation 1/10 (P/N) ratio. The data were normalized by the non-treated cells (100% of metabolic activity). Synthesized polymers showed an absence or low toxicity 2 and 4 days after treatment in the presence and absence of serum. No significant difference in metabolic activity was noted between poly (lysine) treated cells, positive control of transfection and polymer treated cells. No impact on cell proliferation was observed after treatment with the different systems (no significant difference in proliferation between poly (lysine) and treated cells) (Fig. 6b). A toxicity of the polymers marked by a decrease in metabolic activity was observed at high P/N ratio correlated with a decrease in cell proliferation (Supplementary information).

3.4. Transfection studies

The transfection efficiency of the polymer complexes was studied using 3T3 fibroblasts. Polyplexes prepared at different polymer/plasmid DNA ratios ranging from 1/2 to 1/20 (P/N) were used. Polyplexes were incubated with the cells in the presence and absence of 10% serum for 4 h. Non-transfected cells and cells treated with plasmid alone were used as negative controls. Complexed linear poly-L-lysine and complexed linear poly (dimethylaminoethyl methacrylate), similar in structure to the linear polymers studied here, were used as positive controls. Poly-L-lysine with a molecular weight between 70,000 and 150,000 and 1/15 (P/N) was selected because of the highest transfection results obtained using these criteria [33] as poly (dimethylaminoethyl methacrylate with 1:18, 1:22, 1:27, 1:31, 1:36 (P/N) due to their highest transfection ability at these ratios and molecular weight. Luciferase activity observed after treatment with the homopolymer (Fig. 7) was not significant compared with the luciferase activity obtained after

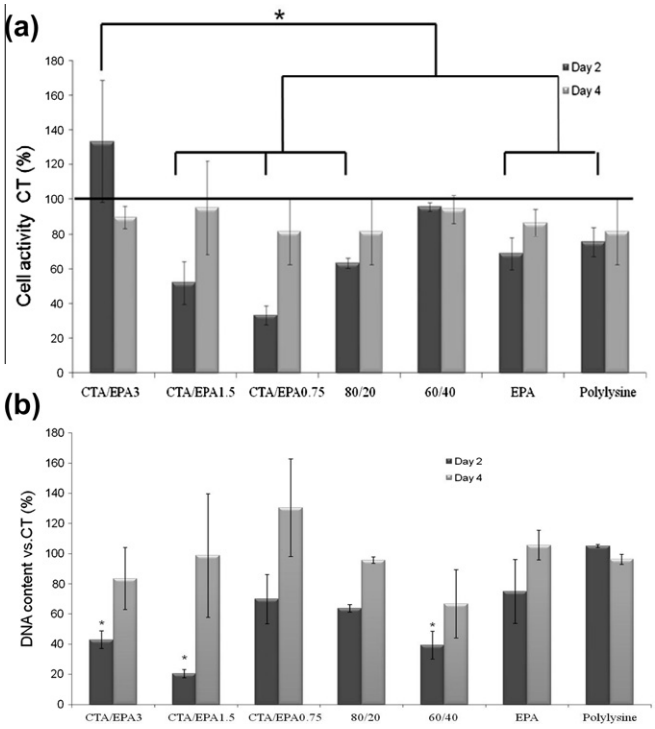


Fig. 6. Cytotoxicity of the polymer/DNA polyplexes at 1:10 P/N ratio (days 2 and 4): (a) cell activity vs. CT (%) (b) DNA content vs. CT (%). *Denotes significant difference between the groups (One-way ANOVA, $p < 0.05$).

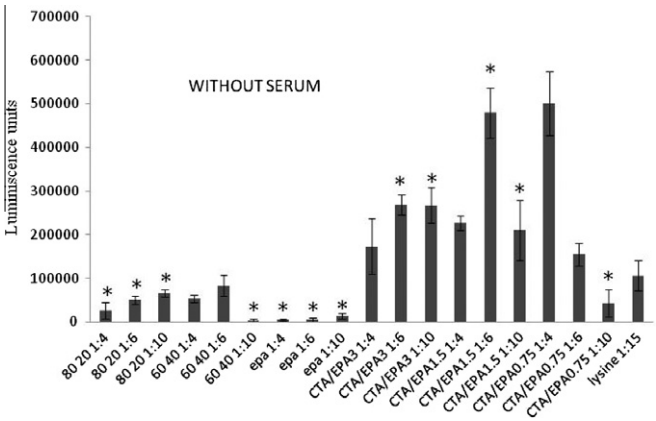


Fig. 7. Transfection efficiency of polymer/DNA polyplexes in 3T3 in comparison with (poly-L-lysine/DNA polyplexes without serum at day 2. *Denotes significant difference between the groups (one-way ANOVA, $p < 0.05$).

treatment with the copolymers, oligomers and poly-L-lysine. This lower transfection ability can be attributed to the lack of total complexation between the homopolymer and DNA until 1/10 ratio (P/N). From this ratio up to 1/20 (P/N), due to an increase in toxicity, no significant differences in terms of transfection efficiency were observed (Fig. 6). In case of the copolymers, the luciferase activity obtained was higher than the homopolymer but did not reach the activity obtained for the positive control (polylysine). No significant differences in luciferase activity were found between the 80/20 and 60/40 compositions.

The highest luciferase activity in 3T3 fibroblasts was obtained with oligomers from the ratio 1/6 to 1/10. This phenomenon may be due to several factors such as the low molecular weight and the introduction of primary amines. Another reason could be the

increasing hydrophobicity of the oligomers (due to the introduction of a hydrophobic chain in the structure) in comparison with copolymers (more hydrophilic). Anderson et al. observed similar results for carriers based on linear poly (ester amine)s [34]. This can be accounted for by the presence of segments in the polymers with a higher hydrophobicity improves cellular uptake through hydrophobic interactions with cell membranes. The second possibility is that polyplexes of more hydrophobic polymers possess better biophysical properties, including smaller polyplex size, easier polyplex dissociation and better endosomal escape increasing the transfection efficiency.

Another interesting feature of this type of oligomers is its ability to mediate higher transfection in the presence of serum as can be seen in Fig. 8 where an increase in transfection in all the ratios was observed except in the telomer CTA/EPA 3, poly-L-lysine and poly (dimethylaminoethyl methacrylate) which decreased significantly. This phenomenon is also observed with other transfection agents such as cationic polymers or lipids for which the serum in the transfection medium dramatically reduces transfection efficiency [35]. In contrast, PEI and cationic liposome complexes have been shown to be more efficient in vitro and in vivo when formulated with human serum albumin (the most abundant protein in plasma) [36,37]. Nevertheless, why the transfection ability in the presence of serum increases is still unknown. It has been suggested a receptor-mediated mechanism involving the albumin [38,39] and its ability to promote membrane fusion under acidic conditions is at the origin of this increase [40].Thus, the partial protonation of albumin at endosomal pH and its subsequent interaction with the endosome membrane may be involved in the destabilization of the latter. The formation of small, compacted structures is undoubtedly an important feature to mediate high transfection efficiency in the presence of serum, as shown for cationic lipid – or polylysine – based systems [41]. Our hypothesis is that the remaining positive charge of the "oligoplexes" (high P/N ratios: 1:8, 1:10, 1:15, 1:20) interacts with the albumin (primary amines confer them an extra ability to interact with this protein) and consecutively they have more affinity for the albumin in comparison with polylysine and pDMAEMA. It is likely that the presence of the albumin on the oligoplexes minimizes their interaction [42] with other serum components, including oleic acid and heparin, which were shown to promote the dissociation of genetic material from the complexes [43].

These interactions between oligomer-albumin and other proteins present in the plasma such as fibrinogen, fibronectin and heparin are being now evaluated by Quartz Crystal Microbalance (QCM-D) in our laboratory.

It is also noteworthy that it is necessary to use greater quantities (μg) of PDMAEMA polymer than the oligomers to reach similar or higher transfections in the presence of serum. For example, 1:22 and 1:31 (P/N) ratios of the PDMAEMA correspond to 1 μg of DNA and 10 μg and 14 μg of the polymer, respectively, while the 1:6 and 1:10 (P/N) ratios of the CTA/EPA 1.5 correspond to 1 μg of DNA and 3 μg and 5 μg of the polymer, respectively. Judging by these results, their use in vivo applications looks very promising.

3.5. Blood compatibility

3.5.1. Haemolysis of the oligomers

In vitro analysis of red blood cell hydrolysis is a reliable method of determining the suitability of our carrier for gene delivery. During this analysis, PBS and TritonX (detergent) were used as 0% and 100% of haemolytic agents, respectively. The telomers showed a very low level of haemolysis (below 1%) (Fig. 9). A significantly higher haemolysis observed with the polymer CTA/EPA 0.75, haemolysis percentage staying, however, remained very low. These results make them good candidates for potential application as injectable blood stream agents.

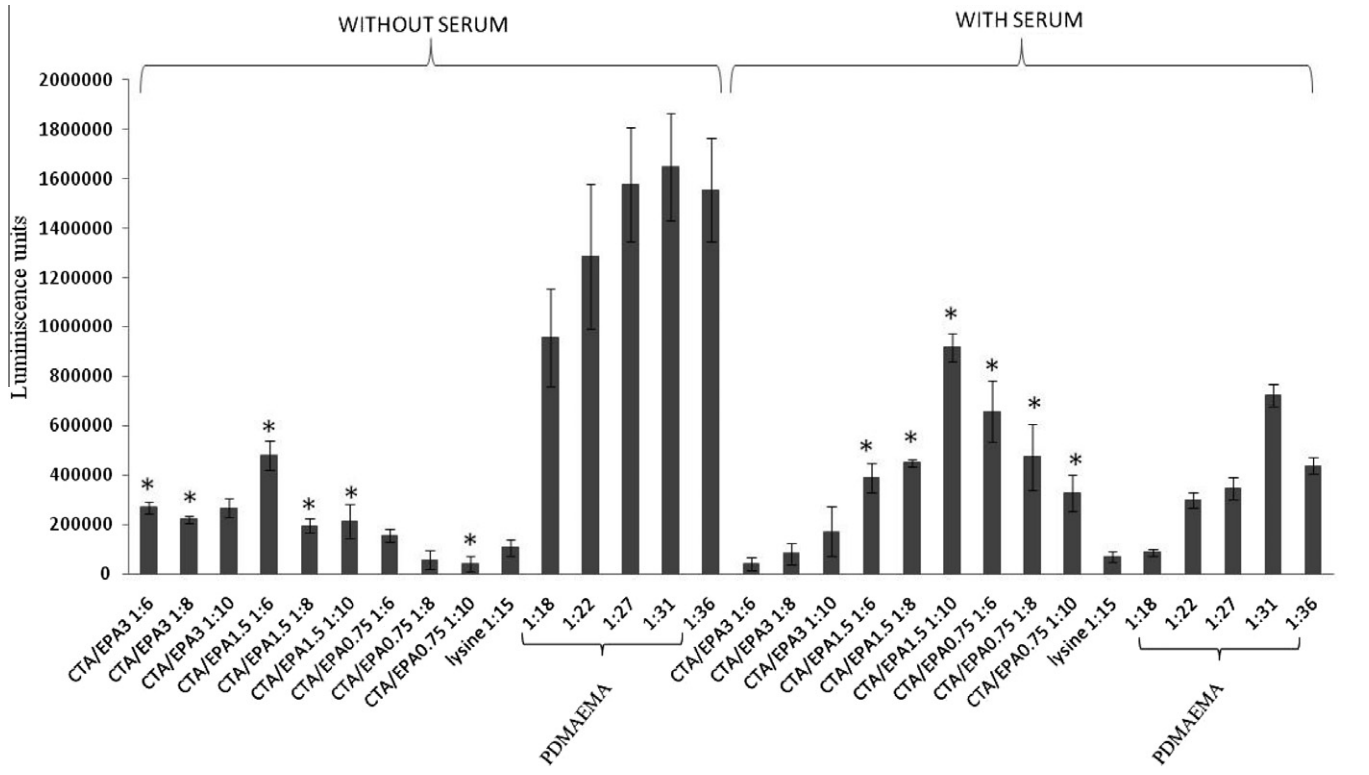


Fig. 8. Transfection efficiency of polymer/DNA polyplexes in 3T3 in comparison with (poly-L-lysine), (poly dimethylaminoethyl methacrylate)/DNA polyplexes with and without serum at day 2. *Denotes significant difference between the groups (one-way ANOVA, $p < 0.05$).

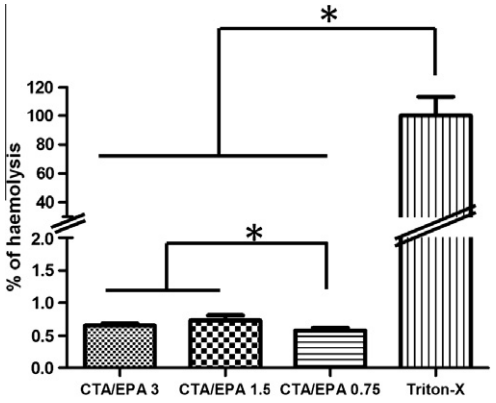


Fig. 9. Hemolysis percentage of red blood cells following incubation of whole blood with oligomers. *Denotes significant difference between the groups (one-way ANOVA, $p < 0.05$).

4. Conclusions

New linear, non-toxic and non-crosslinked polymer systems based on acrylic derivatives or pyrrolidine with two different ionizable groups (tertiary amines as side chains and primary amine as chain end) were prepared as homopolymers, copolymers and oligomers, respectively. Polymer/DNA complexes (polyplexes) were characterized by agarose gel electrophoresis, light scattering and transmission electronic microscopy (TEM). Cell viability and proliferation after contact with polymer and polyplexes were studied by AlamarBlue™ and Picogreen® assays, which showed good cito and hemo-compatibility. These polymers have shown potential application as gene vectors with transfection levels, in some cases, higher than those of commercial poly-lysine and synthetic poly (dimethylaminoethyl methacrylate). Telomers prepared by the free radical polymerization of monomers in the presence of cysteamine present low molecular weight, low polydispersity and one ionizable primary amine group at the end of the chain, together with the tertiary amine groups as side substituents of the acrylic polymer system. These oligomers give an attractive transfection values for serum-sensitive cell lines, which overcomes the limitations associated with the use of highly positively charged polymers. These resorbable polymers have a controlled composition and microstructure with a modulate molecular weight and therefore are very attractive for applications in gene therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2011.06.004.

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