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PEGylated bioreducible poly(amido amine)s for non-viral gene delivery

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ABSTRACT

A facile method for PEGylated bioreducible poly(amido amine)s is described by a one-pot Michael-type addition polymerization of *N*, *N*′-cystaminebisacrylamide (CBA) with a mixture of 4-amino-1-butanol (ABOL) and mono-*tert*-butoxycarbonyl (Boc) PEG diamine. By this approach, two Boc-amino-PEGylated p(CBA-ABOL) copolymers were obtained with the PEG/ABOL composition ratio of 1/10 (**1a**) and 1/6 (**2a**), respectively. These copolymers were characterized by ^{1}H NMR and gel permeation chromatography. The PEGylated copolymers **1a**, and its deprotected analog **1b** with a terminal amino group at the PEG chain, were further evaluated as gene delivery vectors. The copolymers **1a** and **1b** condense DNA into nano-scaled PEGylated polyplexes (<250 nm) with near neutral (2–5 mV, **1a**) or slightly positive (9–13 mV, **1b**) surface charge which remain stable in 150 mM buffer solution over 24 h. UnPEGylated polyplexes from p(CBA-ABOL), however, are relatively less stable and increase in size to more than 1 μm. The PEGylated polyplexes showed very low cytotoxicity in MCF-7 and NIH 3T3 cells and induced appreciable transfection efficiencies in the presence of 10% serum, although that are lower than those of p(CBA-ABOL) lacking PEG. The lower transfection efficiency of the PEGylated p(CBA-ABOL) polyplexes is discussed regarding the effect of PEGylation on endosomal escape of the PEGylated polyplexes.

1. Introduction

The development of safe and effective gene delivery vectors is an essential step for the success of gene therapy [1]. Non-viral vectors, particularly cationic polymers, receive much attention because they have the advantage over viral vectors of relatively low immunogenicity *in vivo* and easy manufacturing [2–6]. Cationic polymers like polyethylenimine (pEI) condense DNA into nanosized and positively-charged polymer/DNA complexes (polyplexes), which undergo cellular uptake via endocytosis and escape from the endosomes by their buffering effect, thus leading to appreciable transfection efficiency *in vitro*[7]. However, systemic gene delivery of positively-charged polyplexes is seriously hampered by extracellular barriers [8,9]. For example, the binding of negatively-charged blood components to the polyplexes may lead to the formation of aggregates and blood clotting *in vivo*[10]. Besides this, after intravenous administration, the polyplexes are rapidly eliminated from circulation by the organs of the reticuloendothelial system, such as liver or spleen [11–13]. To address these barriers, poly(ethylene glycol) (PEG) modification of cationic polymers has been proven as a useful method. PEGylated cationic polymers condense DNA into PEGylated polyplexes, which show several favorable biophysical properties, such as improved colloid stability and reduced interactions of the polyplexes with blood components and the extracellular membrane surface, thus resulting in prolonged circulation and low toxicity *in vivo*[11,14,15].

For the preparation of PEGylated cationic polymers as non-viral gene vectors, two approaches have been reported. The most widely used method is chemical conjugation of functionalized PEG to cationic polymers, such as pEI and poly(L-lysine), to give PEGylated analogs that have been tested for gene delivery *in vitro* and *in vivo*[15–18]. The second PEGylation strategy is directed to polymerization reactions that are initiated by a terminal functional group (e.g. amino group) of PEG [19–23]. In this way, well-defined block copolymers, such as PEG-poly(L-lysine) (PEG-pLL) and pEI-PEG-pEI, were generated [21,23].

We previously reported that poly(amido amine)s with disulfide linkages in the main chain (*SS*-PAA) have very promising properties for gene delivery, as their polyplexes show efficient transfection with concomitant low cytotoxicity [24–29]. A particular property of these polymers is that they are chemically relatively stable in the extracellular environment, but are prone to degradation in the intracellular environment, leading to facilitated polyplex unpacking, thereby yielding efficient gene delivery [29]. However, for further systemic delivery of these polymers, their *in vivo* gene delivery properties have to be improved. Therefore, a further study was performed to design and evaluate PEGylated *SS*-PAA polymers for non-viral gene delivery.

In this paper, we describe the synthesis of PEGylated poly(amido amine)s by a simple one-pot reaction, making this class of PEGylated

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polymers easily accessible. As a representative, the *SS*-PAA with hydroxybutyl side groups (p(CBA-ABOL)) was selected, since in our previous studies it was shown that this polymer induced significantly higher transfection efficiencies *in vitro* against COS-7 cells than pEI, a golden standard in non-viral transfection [29]. Here, we describe one-pot synthesis of the PEGylated p(CBA-ABOL) copolymers, their characterization and the evaluation of the *in vitro* gene delivery properties of these PEGylated *SS*-PAA in relation to their gene transfection in MCF-7 and NIH 3T3 cells.

2. Materials and methods

2.1. Materials

All reagents, *N*, *N*′-cystaminebisacrylamide (CBA, Polysciences, USA), 4-amino-1-butanol (ABOL, Aldrich), glutathione (GSH, Aldrich), trifluoroacetic acid (TFA, Aldrich), propidium iodide (PI, 1.0 mg/mL, Aldrich), Alamar Blue (Biosource, 10× solution, Etten-leur, NL) and α-*tert*-butoxycarbonylamino-ω-amino-terminated poly(ethylene glycol) (BocNH-PEG-NH_2) (3.0 kDa, Aldrich) were purchased in the highest purity and used without further purification. Linear polyethylenimine (ExGen 500, M_w 25 kDa) was ordered from Fermentas (Germany). The plasmid pCMV-GFP, containing a GFP gene under control of a CMV promoter, was purchased from Plasmid Factory (Bielefeld, Germany). The reference polymer p(CBA-ABOL) (M_w 3.2 kDa) was synthesized by Michael addition polymerization of ABOL to equimolar monomeric ratio of CBA as previously reported [29].

2.2. Synthesis of PEGylated bioreducible poly(amido amine) copolymers

PEGylated p(CBA-ABOL) copolymers **1a** and **2a** were synthesized by Michael-type addition of *N*, *N*′-cystaminebisacrylamide (CBA) and a mixture of mono-*tert*-butoxycarbonyl PEG diamine (BocNH-PEG-NH_2) and 4-amino-1-butanol (ABOL) at various molar ratios (*i.e.* 2/98 or 5/95), as shown in Scheme 1. In a typical experiment on the synthesis of **1a**, CBA (1.06 g, 4.07 mM), ABOL (0.36 g, 3.99 mM), and BocNH-PEG-NH_2 (0.24 g, 0.08 mM) were added into a brown reaction flask and dissolved in methanol/water mixture (1.1 mL, 4/1, v/v). Polymerization was carried out in the dark at 45 °C under nitrogen atmosphere. The reaction mixture became homogeneous in less than 2 h due to oligomer formation. The reaction was allowed to proceed for 6 days yielding a viscous solution. Subsequently, 10 mol% excess ABOL (0.036 g, 0.4 mM) was added to consume any unreacted acrylamide groups and stirring was continued for 2 days at 45 °C. The resulting solution was diluted with 30 mL water, acidified with 6 M HCl aqueous solution to pH ~ 3, and then purified by ultrafiltration operation (3000 g/mol cut-off) with acidic deionized water (pH ~ 4). The resultant copolymer **1a** in its HCl-salt form was collected as solid after freeze-drying (0.58 g, yield: 35%).

Amino-terminated PEGylated p(CBA-ABOL) copolymer **1b** was prepared by the acid removal of Boc-protective group in copolymer **1a** (Scheme 1). The deprotection of the amino groups of **1a** (200 mg) was performed in a mixture of methanol/TFA (10 mL, 1/1, v/v) for 8–12 h. After the signals of the Boc-groups have disappeared in the ^{1}H NMR spectra, the solution was diluted with water, adjusting to pH ~4 with 4 M NaOH and then purified by ultrafiltration (1000 g/mol cut-off) with acidic deionized water (pH ~4). The copolymer **1b** was collected as its HCl-salt form after freeze-drying (yield: 86%).

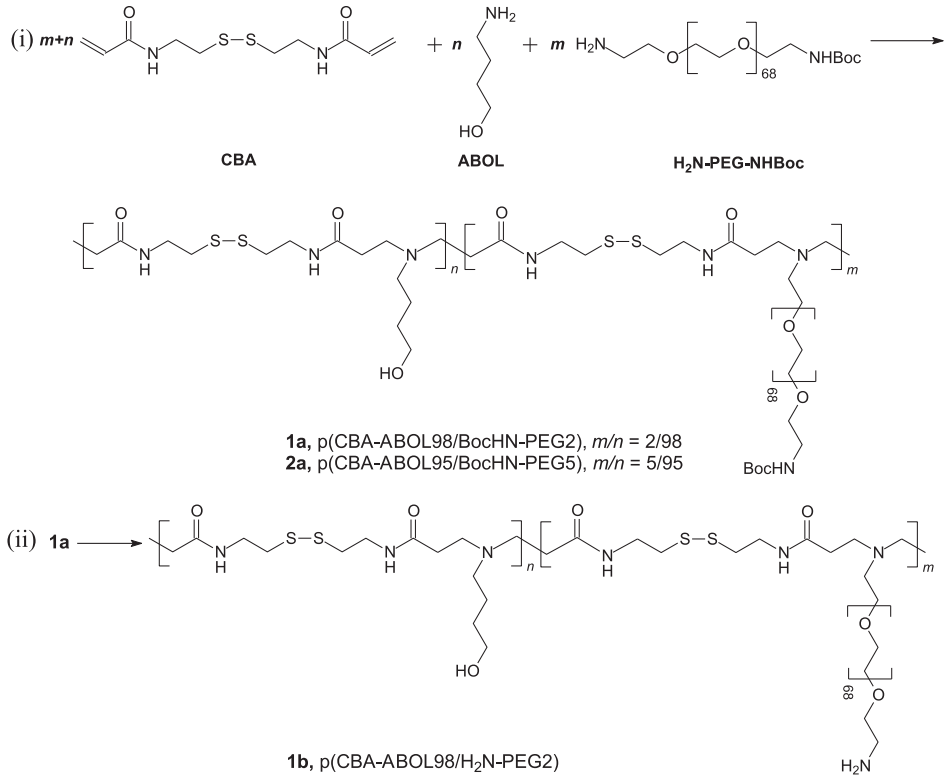
2.3. Polymer characterization

2.3.1. ^{1}H NMR

The ^{1}H NMR spectra of the synthesized PEGylated p(CBA-ABOL) copolymers (in D_2O) were recorded on Varian Inova spectrometer operating at 300 MHz.

2.3.2. Gel permeation chromatography (GPC)

The molecular weight and polydispersity of copolymers **1a** and **2a** were determined by GPC relative to PEO standards (Polymer Labs). In short, GPC measurements were performed using a PL-GPC 120



Scheme 1. Synthesis of (amino-terminated) PEGylated p(CBA-ABOL) copolymers.

Integrated GPC/SEC System (Polymer Lab, USA) equipped with one PL aquagel-OH 30 and one PL aquagel-OH 40 column (8 μm, 300×7.5 mm, Polymer Labs). Data were collected using a differential refractometer. 0.3 M NaAc aqueous solution (pH 4.4) plus methanol (70/30, v/v), was used as eluent at a flow rate of 0.5 mL/min.

2.3.3. Acid–base titration

The buffering capacity of PEGylated p(CBA-ABOL) copolymers **1a** and **2a**, and that of the unPEGylated reference polymer p(CBA-ABOL), were determined by acid–base titration. Therefore, a known amount of the polymer, which was isolated as its HCl salt with 0.025 mmol of protonable amine groups, was dissolved in 5 mL of 150 mM NaCl aqueous solution. The pH of the polymer solution was set at 2.0 and the solution was titrated with 0.01 M NaOH solution using an automatic titrator (Metrohm 702 SM Titrino). The buffering capacity is defined as the percentage of (protonable) amine groups becoming protonated from pH 7.4 to 5.1, and can be calculated from equation:

$$\textit{Buffer capacity}\ (\%) = 100(\Delta V_{NaOH} \times 0.01M) / N\,mol$$

wherein ΔV_{NaOH}, is the volume of NaOH solution (0.01 M) required to bring the pH value of the polymer solution from 5.1 to 7.4, and *N mol* (0.025 mmol), is the total moles of protonable amine groups in the known amount of PEGylated *SS*-PAA copolymer [29].

2.4. Particle size and zeta-potential measurements

Polyplexes at various polymer/DNA mass ratios (24/1–96/1) were prepared by adding a HEPES buffer solution (20 mM, pH 7.4, 5% glucose) of polymer (800 μL, 360–1440 μg) to a HEPES buffer solution (20 mM, pH 7.4, 5% glucose) of plasmid DNA (200 μL, 15 μg), followed by vortexing for 5 s and incubating at room temperature for 30 min. The particle size and surface charge of polyplexes were measured at 25 °C with a Nanosizer Instrument (Malvern Instruments Ltd., Malvern, UK).

To evaluate the colloidal stability of the polyplexes of copolymers **1a** and **1b**, the same experimental procedure as mentioned above was applied to the preparation of the polyplexes at various mass ratios, but in a HEPES buffer solution (20 mM, pH 7.4) without glucose. Next, 0.1 mL of saline solution (165 mM) was added to give a final salt concentration of 150 mM in the resultant polyplex solution (1.1 mL), and the solution was incubated at 37 °C. The particle size of polyplexes at different incubation times (0.5 h, 4 h and 24 h) was determined at 25 °C with a Nanosizer Instrument (Malvern Instruments Ltd., Malvern, UK).

2.5. Agarose gel retardation

Polyplexes were made by adding 10 μL of polymer solution (various concentrations in 20 mM HEPES buffered saline (HBS)) to 10 μL plasmid solution (80 μg/mL in HBS buffer pH 7.4), followed by vortexing for 5 s and the dispersions were incubated for 30 min at room temperature. Next, 10 μL of HBS buffer solution (as control) or HBS buffer containing glutathione (GSH) was added to give final 5.0 mM GSH in the resultant solution, and the dispersions were incubated for 30 min. After addition of 5 μL of 6× loading buffer containing bromophenol (Fermentas), 10 μL of this mixture was loaded onto a 0.7% agarose gel containing 0.5 μg/mL ethidium bromide at 100 V for 60 min in TAE buffer (40 mM, tris-acetate, 1 mM EDTA). After development of the gel, DNA was visualized with a UV lamp using Gel Doc 2000 (Bio-Rad).

2.6. In vitro transfection and cell viability assays

2.6.1. Transfection experiments

Transfection experiments were carried out on MCF-7 and NIH 3T3 cell lines between passages 3 and 8 using a pCMV-GFP reporter plasmid. Cells were cultured in DMEM full medium containing 10% FBS and penicillin/streptomycin (10,000 U/mL) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated in 6-well plates at 1.5×10^5 and 1×10^5 cells/well for MCF-7 and NIH 3T3 cells, respectively. The cells were cultured in 2 mL DMEM full medium until about 70% confluency was reached. They were then washed twice with fresh 1× PBS buffer and incubated again in 2 mL fresh DMEM full medium for gene transfection. Next, PEGylated p(CBA-ABOL) copolymer/plasmid DNA weight ratio of 96/1 was used to prepare polyplexes. In brief, the polyplexes were prepared by adding a HEPES buffer solution (20 mM, pH 7.4, 5% glucose, 200 μL) of the copolymer (2400 μg/mL) to a HEPES buffer solution (20 mM, pH 7.4, 5% glucose, 50 μL) of plasmid DNA (100 μg/mL), followed by gentle shaking and incubation at room temperature for 30 min. In a transfection experiment, the cells were incubated with desired amounts of polyplexes (e.g. 200 μL polyplexes solution with 4 μg plasmid DNA per well) for 6 h or 24 h at 37 °C in a humidified 5% CO_2-containing atmosphere. The incubation of the polyplexes with the cells was performed in the presence of 10% serum. Afterwards, the medium was replaced with 2 mL of fresh DMEM full medium and the cells were allowed to incubate further for a total of 44 h. All transfection experiments were carried out in triplicate. A pEI/DNA (ExGen 500) and a p(CBA-ABOL)/DNA formulation, prepared at a nitrogen/phosphate (N/P) ratio of 6/1 (2 μg DNA) and a polymer/DNA mass ratio of 48/1 (2 μg), respectively, were used as positive controls in the transfection experiments by using the same experiment operations (24 h transfection time) as mentioned above.

2.6.2. Cell viability assay

The number of viable cells was measured using an Alamar Blue assay. In detail, the medium was discarded and the cells (including untreated cells, *i.e.* cells not exposed to transfection systems) were washed twice with fresh 1× PBS buffer and incubated with freshly made 2 mL of 1× Alamar Blue–DMEM media (*i.e.* 10-fold diluted Alamar Blue solution in DMEM full medium) for 4 h. Then, 200 μL of the media from each well (and also 1× Alamar Blue–DMEM media as a blank) was transferred to a 96-well plate for fluorescence recording. The fluorescence intensities were recorded using a fluorescence spectrophotometer (Perkin Elmer LS50B) with excitation and emission wavelengths of 545 and 590 nm, respectively. Cell viability was calculated according to the following equation:

$$\text{Cell viability}\ (\%) = \left(F_{sample} - F_0\right) / \left(F_{control} - F_0\right) \times 100$$

wherein F_{sample}, $F_{control}$, and F_0 represent the fluorescence density of the medium of transfected cells, the medium of untreated cells, and 1× Alamar Blue–DMEM medium as a blank, respectively. The calculated value for untreated cells as a control was taken as 100% cell viability.

2.6.3. Flow cytometry

After Alamar Blue assay, transfection efficiency of the polyplexes of the copolymers against MCF-7 and NIH 3T3 cell lines was determined by flow cytometry. In detail, after the cell viability assay, the Alamar Blue–DMEM media was discarded and the cells were washed twice with 1× PBS buffer, trypsinized and transferred to sterile tubes followed by centrifugation at 300 *g* for 5 min. The supernatant was poured off, and the cells were resuspended in 0.4 mL of 1× PBS containing 1% FBS. Then, 10 μL of propidium iodide (PI, 1.0 mg/mL) was added to the cells to determine cell viability. Fluorescence for both GFP and PI were detected using a FACScan flow cytometer (BD) with excitation wavelengths of 488 and 535 nm, respectively. The cytometer was calibrated with a negative control (untreated cells) to identify viable cells and blank transfection efficiency. The percentages of transfected cells were quantified from a gated viable population of

10,000 cells. In all experiments, the viable cells were gated based on cell exclusion of PI.

2.6.4. Transfection experiments in the presence of chloroquine

The influence of chloroquine on the transfection efficiency of the polyplexes of the copolymer **1b** at a polymer/DNA mass ratio of 96/1 was examined by using the same procedure as mentioned in Section 2.6.1; in this case MCF-7 cells were incubated with the polyplexes in the presence of chloroquine (final conc. 100 μM) for 6 h and were allowed to incubate further for a total of 44 h. Cell viability and transfection efficiency were determined as described in Sections 2.6.2 and 2.6.3, respectively.

2.7. Statistical analysis

Comparisons between two samples were performed with the Student's *t*-test. Differences were considered to be statistically significant at $p<0.05$.

3. Results and discussion

3.1. Synthesis and characterization of PEGylated bioreducible poly(amido amine)s

The PEGylated p(CBA-ABOL) copolymers **1a** and **2a** could be readily synthesized in a one-pot reaction by Michael-type addition polymerization of *N*, *N*′-cystaminebisacrylamide (CBA) with an appropriate mixture of the primary amino monomers 4-amino-1-butanol (ABOL) and mono-*tert*-butoxycarbonyl (Boc) PEG diamine (BocNH-PEG-NH_2) (Scheme 1). To introduce the PEG chain into the p(CBA-ABOL) polymer, two different molar feed ratios (*i.e.* 2/98 or 5/95) of BocNH-PEG-NH_2 and ABOL were applied. Since the Michael-type addition reaction is a stepwise process, equal molar amounts of CBA monomer and amine monomer (*i.e.* total amount of ABOL and BocNH-PEG-NH_2) were used in the reactions to give the copolymers **1a** and **2a** of highest theoretical molecular weight. After 6 days of reaction at 45 °C in a methanol–water mixture (4:1 v/v) under nitrogen atmosphere, any residual acrylamide groups were eliminated by addition of excess of ABOL and further reacted for two days. The copolymers were isolated as their HCl salt by ultrafiltration (3000 g/mol cut-off) and freeze-drying. They showed good solubility in the pH range 2–11. The ^{1}H NMR spectra of these copolymers were in full accordance with the expected structures (Fig. 1a). The absence of any proton signals between 5 and 7 ppm indicates that no residual acrylamide end groups were present in these copolymers. The composition ratios of PEG moieties and ABOL in the copolymers were determined by comparing the integrals of the ^{1}H NMR signals at δ3.75 and δ1.52, respectively, attributed to the methylene protons ($\underline{CH_2}\underline{CH_2}O$) in the PEG moiety and the methylene protons ($\underline{CH_2}CH_2OH$) in the ABOL moiety (Fig. 1a, Table 1). Compared to the PEG/ABOL monomer feed ratios, higher PEG/ABOL composition ratios in the products were obtained. For example, the molar PEG/ABOL composition ratio determined for copolymer product **1a** was 1/10, which is much higher than the feed ratio of 2/98 (*i.e.* 1/49) in the synthesis (Table 1). The relatively high PEG content in the eventually isolated products can be explained by the preferential removal of the (low-molecular weight) unPEGylated p(CBA-ABOL) polymer fraction from the product mixture during the ultrafiltration process. Thus, only polymers that contain at least one PEG chain ((M_w 2.9 kDa) are retained after ultrafiltration. The relative molecular weights (PEO standard) and polydispersity of the copolymers **1a** and **2a** were determined by gel permeation chromatography (GPC) measurement (Table 1). Fig. 2 shows typical GPC traces of these copolymers and the starting reactant BocNH-PEG-NH_2 as a control. The peak of copolymer **1a** exhibited a unimodal distribution

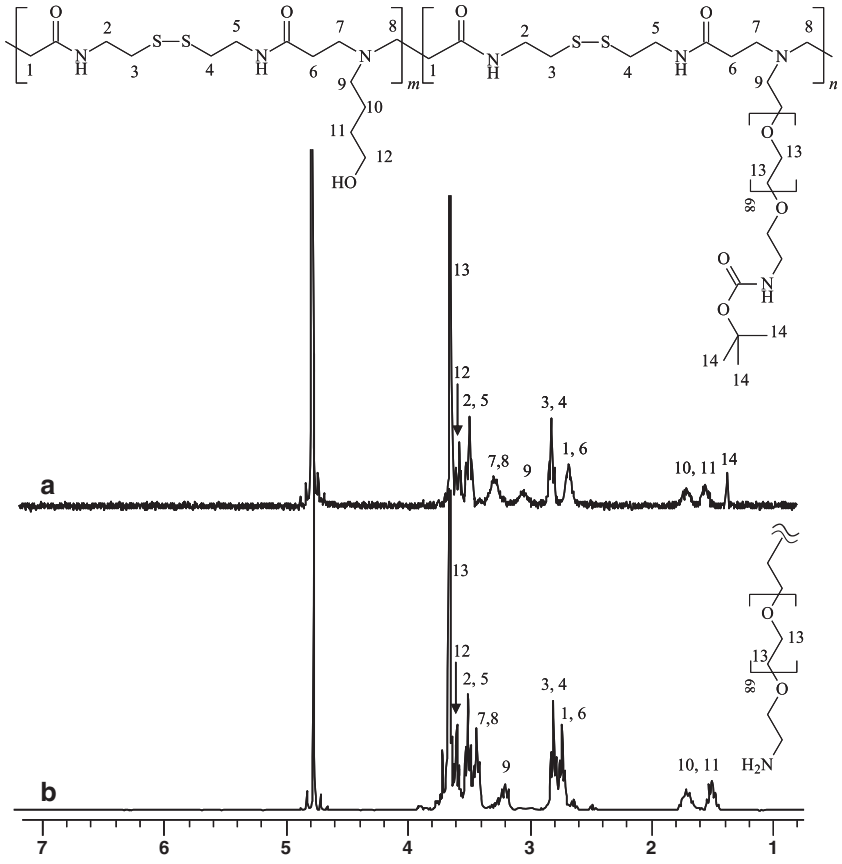


Fig. 1. ^{1}H NMR spectra (D_2O, 300 MHz) of (a) p(CBA-ABOL98/BocNH-PEG2) (**1a**) and (b) p(CBA-ABOL98/H_2N-PEG2) (**1b**).

Table 1
Characteristics of PEGylated p(CBA-ABOL) copolymers **1a** and **2a**: composition, weight-average molecular weight (M_w), and polydispersity index (M_w/M_n).

Polymer #	Polymer acronym	Feed ratio PEG/ABOL (*m*/*n*, mol/mol)	Composition PEG/ABOL (*m*/*n*, mol/mol)	M_w (kDa)	M_w/M_n
1a	p(CBA-ABOL98/BocNH-PEG2)	2/98	1/10	5.7	1.46
2a	p(CBA-ABOL95/BocNH-PEG5)	5/95	1/6	9.3/4.0[a]	1.1/1.2[a]

[a] M_w and polydispersity index of two peaks.

in its GPC trace with an earlier elution time than BocNH-PEG-NH_2 and weight molecular weight (M_w 5.7 kDa) that is about two times higher than that of BocNH-PEG-NH_2 (M_w 2.9 kDa by GPC). These results and the NMR data suggested the successful synthesis of the PEGylated copolymer **1a** and the absence of unPEGylated p(CBA-ABOL) or unreacted BocNH-PEG-NH_2 in this product. Product **2a** showed two distributions in its GPC trace. One of the two peaks appeared at the same position of the copolymer **1a**, but the other peak (M_w 9.3 kDa) appeared at earlier elution time. These results suggest that in product **2a** two types of PEGylated p(CBA-ABOL) copolymers might be present.

On the basis of the above data, PEGylated p(CBA-ABOL) copolymer **1a** was used for further study. By the acid removal of the Boc-protective group in the copolymer **1a**, amino-terminated PEGylated p(CBA-ABOL) copolymer **1b** was obtained (Scheme 1). The absence of a signal for the methyl protons of the Boc group at δ 1.45 ppm indicates that the protective group has been totally removed (Fig. 1b). Due to the presence of protonable primary amino group at the terminal position of the PEG chain in copolymer **1b**, it was expected that the copolymer **1b** could show different gene vector properties compared to copolymer **1a**, containing the hydrophobic Boc group at this position (*vide infra*).

3.2. Buffer capacity of PEGylated p(CBA-ABOL)

From previous studies it appeared that a high buffer capacity of poly(amido amine)s, as defined as the percentage of amino nitrogens that becomes protonated in the pH range 7.4–5.1, is positively correlated with a high transfection efficiency [29]. A high buffer capacity is frequently related to facilitated endosomal escape of polyplexes, caused by the proton sponge effect of certain polymers like pEI [7]. However, it should be kept in mind that the increasingly positive charges in the polymer may also induce conformational changes and increased interactions with the endosomal membrane, which also contribute or even govern the endosomal escape process. The buffer capacities of the BocNH-PEGylated p(CBA-ABOL) copolymer **1a**, the H_2N-PEGylated p(CBA-ABOL) copolymer **1b**, and the unPEGylated reference polymer p(CBA-ABOL) were determined by acid–base titration between pH 5.1 and pH 7.4, *i.e.* the relevant region for endosomal acidification. Fig. 3 shows the titration curve of the copolymer **1a**, as a typical example. Both PEGylated copolymers **1a** and **1b** showed very similar acid–base titration curves as the unPEGylated p(CBA-ABOL) homopolymer in the pH range 7.4–5.1. Thus, their buffer capacities are about the same (72%), and are much higher than that of 25 kDa pEI (18.6%). It can be concluded that the presence of the PEG side chain in the copolymers **1a** and **1b** has negligible effect on the protonation behavior of the tertiary amino groups in the polymer backbone and does not impair the proton sponge capacity of these polymers.

3.3. DNA binding and release of PEGylated p(CBA-ABOL) polyplexes

Agarose gel electrophoresis experiments were carried out to study the binding behavior of the copolymers **1a** and **1b** with plasmid DNA. In Fig. 4, it is shown that the H_2N-PEGylated p(CBA-ABOL) copolymer **1b** has stronger capability for DNA binding than the BocNH-PEGylated p(CBA-ABOL) copolymer **1a**, since fully retarded DNA mobility was already achieved at 24/1 ratio, whereas for copolymer **1a** a ratio between 48/1 and 96/1 is needed. This indicates that the protonated amino terminus in the PEG of copolymer **1b**, contributes to the DNA binding in the polyplex. However, both PEGylated copolymers bind DNA weaker than the unPEGylated p(CBA-ABOL) where fully retarded DNA mobility is achieved at 12/1 ratio [29].

In previous studies we have demonstrated that the higher gene transfection efficiency of p(CBA-ABOL)-based polyplexes, compared to those of 25 kDa pEI, is probably due to facilitated DNA release from the polyplexes via the cleavage of the disulfide groups in the intracellular reducing environment [29–31]. Therefore, DNA release from the polyplexes of PEGylated p(CBA-ABOL) copolymers **1a** and **1b** was also investigated by a gel retardation assay in the presence of 5.0 mM glutathione, a reducing agent present in the cellular interior. For these polyplexes, efficient DNA release was observed at and above the polymer/DNA mass ratio of 24/1 (Fig. 4), indicating that facilitated

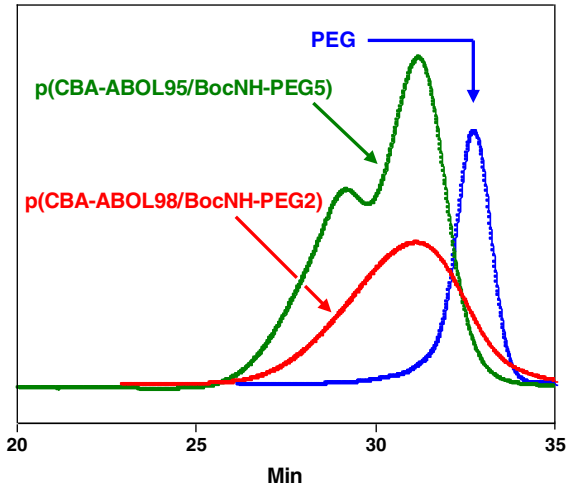


Fig. 2. GPC chromatogram of copolymers: p(CBA-ABOL98/BocNH-PEG2) (**1a**) and p(CBA-ABOL95/BocNH-PEG5) (**2a**), and BocNH-PEG-NH_2 (PEG). The GPC was performed using NaAc buffer (300 mM, pH 4.5, containing 30% (v/v) methanol) as an eluent.

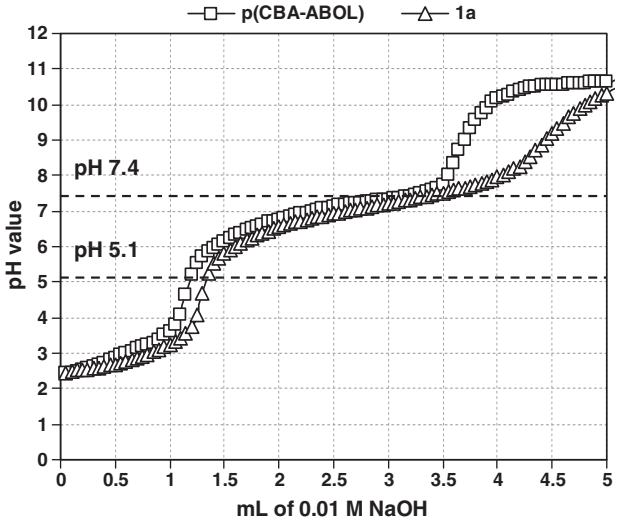


Fig. 3. Titration curves of p(CBA-ABOL98/BocNH-PEG2) copolymer (**1a**) solution (5 mM amino nitrogen atoms, adjusted to pH 2 with 1 M HCl) in 150 mM aqueous NaCl with 0.01 M NaOH. For comparison, the titration curve of reference polymer p(CBA-ABOL) is also presented.

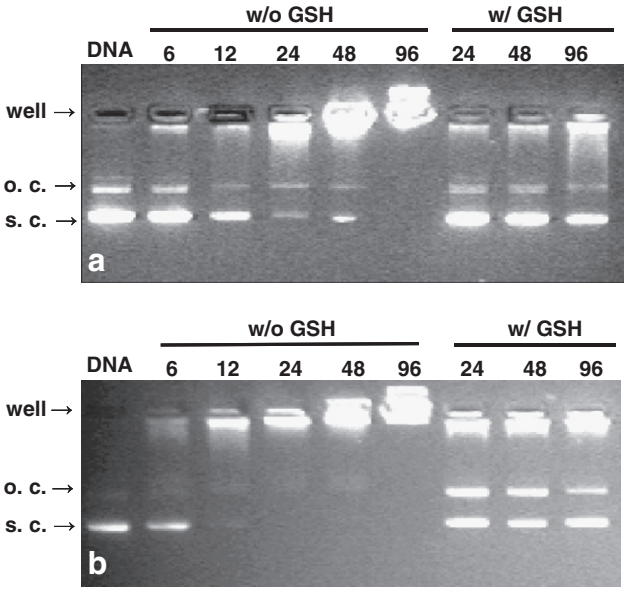


Fig. 4. Gel electrophoresis of PEGylated p(CBA-ABOL)-based polyplexes prepared at different polymer/DNA mass ratios before and after addition of glutathione (GSH, final conc. of 5 mM). (a) p(CBA-ABOL98/BocNH-PEG2) copolymer (**1a**) and (b) p(CBA-ABOL98/H_2N-PEG2) (**1b**). (o.c. = open circular, s.c. = supercoiled form of plasmid DNA).

intracellular DNA release from these polyplexes can be favorably influenced by the cleavage of the disulfide bond in the copolymers.

The polyplexes of copolymers **1a** and **1b** were further studied by dynamic light scattering and zeta-potential analysis, respectively [32]. Both copolymers condensed DNA into nanosized polyplexes (96–109 nm) at the polymer/DNA mass ratios 24/1, 48/1 and 96/1 (Table 2). Moreover, the polyplexes of copolymer **1a** displayed almost neutral surface charge (2–5 mV), which may be attributed to the shielding of the positive surface charge of the p(CBA-ABOL)/DNA complexes by the neutral Boc-terminated PEG chains. For polyplexes of **1b**, slightly positive zeta potentials (9–13 mV) were detected, indicating that these polyplexes present a part of terminal amino groups of PEG at their outer surface. For comparison, at the mass ratio of 48/1 unPEGylated p(CBA-ABOL)/DNA formed polyplexes with zeta potential of ca. 25 mV [29].

The colloidal stability of the polyplexes of copolymers **1a** and **1b**, prepared at the polymer/DNA mass ratios 24/1, 48/1 and 96/1, was monitored by measuring the particle size of the polyplexes in time (Table 2). To mimic the physiological conditions, the ionic strength of the solution of polyplexes was adjusted to 150 mM with NaCl. The polyplexes of these copolymers showed an improved colloidal stability since the size of these polyplexes at various mass ratios remains relatively stable (<250 nm) over 24 h. In contrast, the particle size of polyplexes of unPEGylated p(CBA-ABOL) at the polymer/DNA mass ratio 48/1 rapidly increased from 150 nm to about 1 μm in less than 5 min (data not shown). The improved colloidal stability of the polyplexes of **1a** and **1b** was also supported by the observation that their solutions remained clear over a 24 h period, whereas those of p(CBA-ABOL)-based polyplexes rapidly became turbid.

Table 2
Particle size and surface charge of polyplexes of **1a** or **1b** at different weight ratios.

Polymer #	Weight ratio	Particle size [a] (nm, ±1 nm)	Particle size [b] (nm, ±2 nm)		Zeta potential [a] (mV)
		0.5 h	4 h	24 h	0.5 h
1a	24/1	103	136	176	1.6 ± 0.6
	48/1	103	127	134	3.6 ± 0.4
	96/1	96	183	230	5.0 ± 0.4
1b	24/1	109	118	155	8.8 ± 0.3
	48/1	105	123	165	9.2 ± 1.0
	96/1	101	127	170	12.9 ± 1.0

[a] Determined at 20 mM HEPES, pH 7.4, 5% glucose.
[b] Determined at 20 mM HEPES, pH 7.4, 130 mM NaCl.

3.4. In vitro transfection efficiency and cytotoxicity

The transfection of the polyplexes based on copolymers **1a** and **1b** was studied *in vitro* towards MCF-7 and NIH 3T3 cell lines by using pCMV-GFP as reporter gene. The transfection efficiency was quantified by the percentage of cells expressing green fluorescent protein (GFP), analyzed by flow cytometry. The transfection efficiency of the polyplexes prepared at polymer/DNA mass ratio 96/1 was optimized as a function of the transfection time (6 h or 24 h) against MCF-7 cells in the presence of 10% serum (Fig. 5). It was found that, by increasing the transfection time from 6 h to 24 h, polyplexes of copolymers **1a** and **1b** both induced significantly increased transfection efficiency. Moreover, polyplexes of copolymers **1b** led to statistically higher transfection efficiency as compared to those of **1a** (4.2 ± 0.07% vs. 2.7 ± 0.29%, **1b** vs. **1a**). Moreover, the polyplexes of **1a** and **1b** have very low toxicity (~100% cell viability), as evaluated by Alamar Blue assay (Fig. 5b). Notably, polyplexes of **1a** and **1b** induced lower transfection efficiency (<5% of fluorescent cells) than those of pEI (>5% of fluorescent cells) in

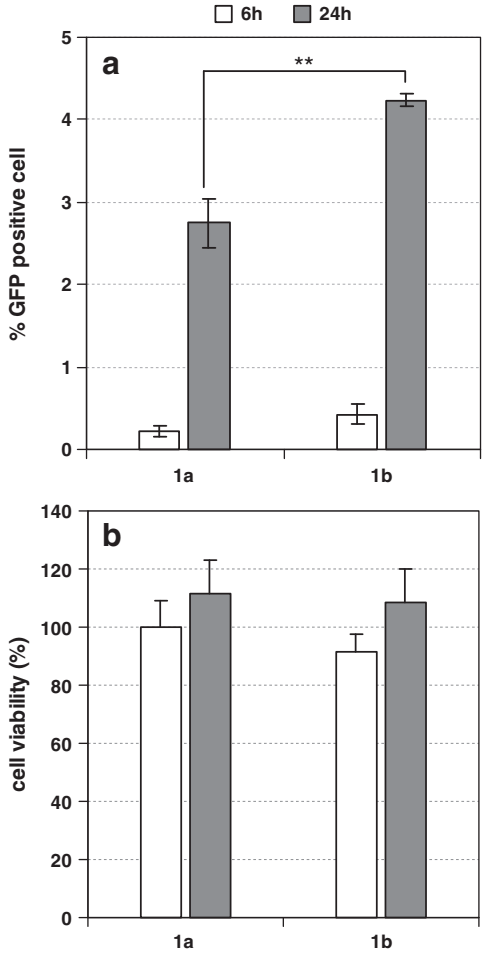


Fig. 5. Transfection efficiencies in MCF-7 cells in the presence of 10% serum (a) and corresponding cell viabilities (b) for the polyplexes of p(CBA-ABOL98//BocNH-PEG2) (**1a**) and p(CBA-ABOL98/H_2N-PEG2) (**1b**), formed at the polymer/DNA mass ratio of 96/1 (4 μg DNA), as a function of transfection time, *i.e.* 6 h (white bars) and 24 h (solid bars). Cell viability was determined by Alamar Blue assay. The data were expressed as mean values (standard deviations) of three experiments. (Student's *t*-test, **$p<0.01$).

MCF-7 and NIH 3T3 cells (Fig. 6a) and also lower than those of unPEGylated p(CBA-ABOL) that yields 30.2 ± 0.4% and 18.5 ± 0.6% positive cells for MCF-7 and NIH 3T3, respectively (data not shown in the figure). Increment of the DNA dose in the polyplexes of copolymer **1b** from 4 μg to 16 μg did not result in increase of the transfection (Fig. 6a). These polyplexes and p(CBA-ABOL)-based polyplexes are essentially non-toxic (~100% cell viability), whereas pEI-based polyplexes are highly toxic (~60% cell viability) (Fig. 6b). One important factor that is responsible for the low transfection efficiency of the polyplexes of **1a** and **1b** at a high DNA dose is likely a poor cell association and internalization, since polyplexes of **1a** and **1b** have much lower surface charge than those of unPEGylated p(CBA-ABOL) and pEI (~+25 mV and +30 mV, respectively). Davis et al. reported that neutral surface of PEGylated polyplexes may impair their efficient cellular association and internalization, resulting in a low gene transfection efficiency [33]. Besides, limited endosomal escape of the PEGylated polyplexes could further impede efficient gene transfection (*vide infra*). Apparently, this is not originating from reduced buffering capacities of the PEGylated polymers, since these polymers have the buffering capacities (72%) as similar as that of the unPEGylated p(CBA-ABOL) and much higher than that of 25 kDa pEI (18.6%). Wagner et al. have recently reported that PEGylated pEI-based polyplexes also showed lower transfection compared to those of unsubstituted pEI, probably due to poor endosomal escape. However, the use of a pH-sensitive linker that released PEG from pEI in the endosomes led to comparable or enhanced transfection efficiencies compared to unPEGylated pEI [34,35].

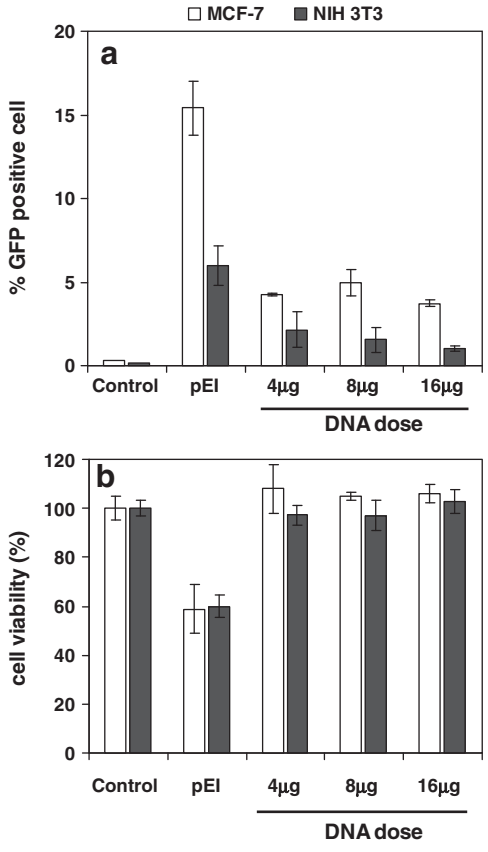


Fig. 6. Transfection efficiencies in MCF-7 cells and NIH 3T3 cells in the presence of 10% serum (a) and corresponding cell viabilities (b) for the polyplexes of p(CBA-ABOL98/H_2N-PEG2) (**1b**), formed at the polymer/DNA mass ratio of 96/1, as a function of DNA dose (4, 8, 16 μg) using 24 h transfection time. The transfection efficiency of pEI (ExGen 500)/DNA polyplexes (2 μg DNA, 24 h transfection time) at an optimal N/P ratio of 6/1 in the presence of 10% serum was taken as a positive control. Transfection experiments using only GFP plasmid were performed as a negative control (bars left). Cell viability was determined by Alamar Blue assay. The data were expressed as mean values (standard deviations) of three experiments.

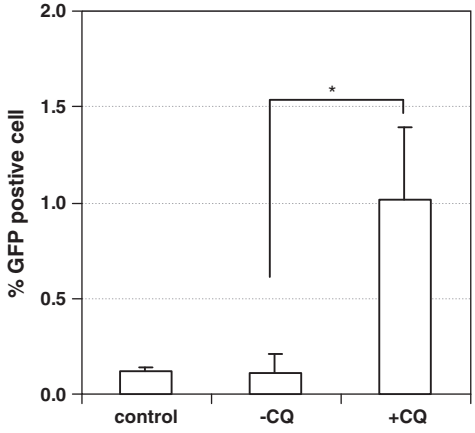


Fig. 7. Transfection efficiency in MCF-7 cells for the polyplexes of p(CBA-ABOL98/H_2N-PEG2) (**1b**), formed at the polymer/DNA mass ratio of 96/1, in the absence and presence of chloroquine (CQ). Transfection experiments using only GFP plasmid were performed as a negative control. The data were expressed as mean values (standard deviations) of three experiments. (Student's *t*-test, *$p<0.05$).

It has been found that chloroquine (CQ) can induce enhanced transfection, which has been attributed to several factors as promotion of endosomal buffering, endosomal disruption, inhibition of lyosomal degradation, and/or facilitated dissociation of DNA from the polyplexes [36]. The data in Fig. 7 show that, for the polyplexes of **1b**, the presence of CQ results in statistically significant higher transfection of MCF-7 cells, while remaining the low cytotoxicity (101.4 ± 9% without CQ vs. 97 ± 4.3% with CQ). The CQ enhancement may serve as an indication that PEGylated polyplexes of **1b** could undergo limited endosomal escape.

Further progress in the development of these non-toxic, bioreducible p(CBA-ABOL) vectors for *in vivo* delivery is expected by introduction of a targeting moiety at the terminal position of the PEG chain, promoting receptor-mediated uptake and a pH-cleavable linker in the PEG-monomer, enabling deshielding of the polyplex in the endosomal compartments [37,38]. Besides, the mechanism of cell uptake of these vectors and intracellular DNA trafficking will also be examined in detail. These will be the subject of further studies.

4. Conclusion

We have demonstrated that a one-pot Michael addition reaction using an amino-terminated PEG as a comonomer in an equimolar mixture of bisacrylate and primary amine monomers is a versatile approach to obtain PEGylated *SS*-PAA copolymers. The PEGylated p(CBA-ABOL) copolymer **1a**, comprising a single PEG side chain to a (CBA-ABOL) polymer has been successfully obtained using an optimal feed ratio between ABOL and PEG. The Boc-protected terminal amino group at the PEG chain could be easily removed, to yield **1b**, possessing a primary amino group at the terminal position of the PEG chain. These PEGylated p(CBA-ABOL) polymers both show good colloidal stability under the physiological conditions, which are important properties for gene delivery *in vivo*. PEGylated p(CBA-ABOL) polyplexes give notably lower transfection than unPEGylated polyplexes of p(CBA-ABOL) or pEI. This is likely caused by limited endosomal escape.

Acknowledgments

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