Random and block copolymers of bioreducible poly(amido amine)s with high- and low-basicity amino groups: Study of DNA condensation and buffer capacity on gene transfection

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Abstract

Poly(amido amine) (SS-PAA) random and block copolymers having bioreducible disulfide bonds in the main chain and amino groups with distinctly different basicity in the side chain were designed and synthesized by Michael addition polymerization between N, N'-cystaminebisacrylamide (CBA) and two amine monomers, i.e., histamine (HIS) and 3-(dimethylamino)-1-propylamine (DMPA). Copolymers containing variable HIS/DMPA ratios show higher ability to bind DNA than p(CBA-HIS) homopolymer and condense DNA into the polyplexes with particle sizes (<150 nm) that are smaller than polyplexes of p(CBA-HIS) (∼220 nm). The buffer capacities of the copolymers increase with increasing HIS/DMPA ratio. These copolymers are able to transfect COS-7 cells in vitro with efficiencies that increase with increasing HIS/DMPA ratio. The random and block copolymers at a HIS/DMPA ratio of 70/30 combines optimal DNA condensation capability and buffer capacity, thereby inducing higher transfection efficiency in the absence and presence of serum as compared to p(CBA-HIS) homopolymer. Moreover, random and block copolymers show a similar transfection capacity, but both have higher capacity than the physical mixtures of p(CBA-HIS) and p(CBA-DMPA) homopolymers. XTT assay reveals that the polyplexes of the SS-PAA copolymers have essentially low cytotoxicity when the highest transfection activity is observed.
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1. Introduction

The development of safe and efficient gene delivery vectors is an essential requisite for the advancement of gene therapy [1,2]. Non-viral vectors particularly cationic polymers receive much attention because they have advantages over viral vectors with low immunogenicity in vivo and easy manufacturing [3–5]. To achieve successful gene delivery, polymer-based vectors must overcome a series of extra- and intracellular barriers that include stability of vector and DNA in the extracellular space, cellular uptake, endosome escape, cytosolic transportation, effective vector unpacking and nuclear translocation [6,7]. In the past decades, many polymer systems such as poly(L-lysine) (pLL) and poly(ethylenimine) (pEI) have been studied as non-viral vectors for gene delivery. However, compared to viral systems,

these vectors are hampered by relatively low transfection efficiency [8]. Efforts to improve the low efficiency are directed to polymeric vectors that are designed to overcome the aforementioned barriers in gene delivery. Important properties of polymeric vectors are DNA condensation ability and buffer capacity, which is the capacity of the polymer to bind protons when the pH in the endosomes decreases from pH 7.4 to 5.1 [9]. A high condensation ability of the polymer will induce the formation of nanosized polymer/DNA complexes (polyplexes) that may undergo cellular uptake via endocytosis. A high buffer capacity of the polymer may facilitate endosomal escape of the polyplexes as extensive protonation of the polymer can induce osmolysis, conformational and/or electrostatic changes that cause endosome disruption [10,11]. Moreover, vector unpacking is necessary to trigger efficient gene delivery inside the cell [12]. In relation to these requirements, Midoux et al. coupled histidine to amino residues of pLL and found that histidyl-pLL has improved buffer capacity, inducing higher transfection efficiency than pLL [13]. In another approach, Putnam et al. modified the amino residues of pLL with imidazole groups and showed that the resulting increase of buffer capacity and concomitant decrease of the charge density of the polymer gives high transfection efficiency at substitution of ca. 86.5 mol% imidazole [14]. A recent work reported by Kataoka et al. illustrated that a PEG-poly[(3-morpholinopropyl) aspartamide]-poly(L-lysine) triblock copolymer containing both high- and low-pK_a groups combines high buffer capacity and good DNA condensation ability, thereby yielding a transfection efficiency that is about 10 times higher than pegylated pLL [15]. The introduction of disulfide bonds in the polymeric vector can have a favorable effect on the unpacking of vector inside the cell due to bioreductive cleavage of the disulfide bonds. This is shown by Pichon et al., who achieved a ten-fold higher transfection efficiency for disulfide coupled pLL segments than for pLL [16]. Efficient gene delivery was also observed with bioreducible polypeptides with histidine and lysine residues leading to five-fold higher transfection efficiency compared to pEI [17].

We previously reported on bioreducible poly(amido amine)s (SS-PAAs) that combine efficient gene delivery with a low cytotoxicity profile [18–20]. The SS-PAAs with disulfide linkages in the main chain and various functionalities in the side chain can be readily synthesized by Michael addition between N, N'-cystaminebisacrylamide (CBA) and the appropriate amine compound. The presence of the disulfide linkages makes these polymers degradable inside the reductive environment of the cells, triggering efficient gene release inside cells. Moreover, the side groups in the polymer also have an influence on transfection capacity. The SS-PAA derived from 3-(dimethylamino)-1-propylamine having a dimethyl amino side group (p(CBA-DMPA)) shows much higher DNA condensation capability than the SS-PAA derived from histamine possessing an imidazole side group (p(CBA-HIS)) and condenses DNA into nanosized polyplexes of about 100 nm at low polymer/DNA mass ratios. However, transfection *in vitro* towards COS-7 cells is more effective for p(CBA-HIS) than for p(CBA-DMPA). This may be attributed to the higher buffer capacity of p(CBA-HIS) (pK_a value ~ 6.5) compared to p(CBA-DMPA) (pK_a value ~ 8.0).

In this study, we aimed to investigate SS-PAA copolymers that combine the beneficial properties of high buffer capacity with high DNA condensation capability. Therefore, random and block copolymers of SS-PAA were synthesized containing different amounts of amino side groups with high and low-basicity, *i.e.* dimethylamino (p(CBA-DMPA)) and imidazole (p(CBA-HIS)) side groups, respectively. In these copolymer systems, the optimal combination of buffer capacity and DNA condensation capability was studied. Moreover, the effect of structure of copolymer, *i.e.* random versus block pattern, on the gene delivery properties and transfection efficiency has been studied.

2. Materials and methods

2.1. Materials

All monomers, 3-(dimethylamino)-1-propylamine (DMPA, Aldrich), Histamine base (HIS, Aldrich), N, N'-cystaminebisacrylamide (CBA, Polysciences, USA), were ordered in the highest purity and used without further purification. ExGen 500 (linear polyethylenimine, 22 kDa) was ordered from Fermentas (Germany). The plasmid pCMV-LacZ, containing a bacterial LacZ gene preceded by a nuclear localization signal under control of a CMV promoter, was purchased from Plasmid Factory (Bielefeld, Germany). p(CBA-HIS) and p(CBA-DMPA) were synthesized by Michael addition polymerization of HIS or DMPA to equimolar monomeric ratios of CBA as previously reported [19].

2.2. Synthesis of bioreducible poly(amido amine) (SS-PAAs) copolymers

2.2.1. Random copolymers

SS-PAA random copolymers were synthesized by polyaddition of CBA and a mixture of HIS and DMPA at various ratios (*i.e.* 70/30, 50/50 and 30/70, mol/mol) as shown in Fig. 1a. In a typical experiment, the p(CBA-HIS70/DMPA30) random copolymer was synthesized by adding CBA (1.67 g, 6.41 mmol), HIS (0.51 g, 4.49 mmol) and DMPA (0.20 g, 1.92 mmol) into a brown reaction flask with methanol/water mixture (3.2 mL, 4/1, v/v) as a solvent and stirring the reaction mixture at 45 °C in the dark under nitrogen atmosphere. The reaction was allowed to proceed for 6 days, yielding a viscous solution. Subsequently, 10 mol % excess of DMPA (0.07 g, 0.06 mmol) was added to consume any unreacted acrylamide groups and stirring was continued for 2 days at 45 °C. The resulting solution was diluted with water to about 30 ml, acidified with 6 M HCl to pH ~ 4, and then purified by ultrafiltration (5000 g/mol cut-off) with deionised water adjusted to pH ~ 4 with 0.1 M HCl. After freeze-drying the polymers in their HCl-salt form were collected as a solid powder (0.8 g, 35%). The final composition of the polymers was determined by ^{1}H NMR (D$_2$O, 300 MHz).

2.2.2. Block copolymers

PAA block copolymers with varying ratios of HIS and DMPA (*i.e.* 70/30, 50/50 and 30/70, mol/mol) were synthesized in two steps, as shown in Scheme 1b. First, oligomers of p(CBA-DMPA) with terminal diacrylamide groups and oligomers of
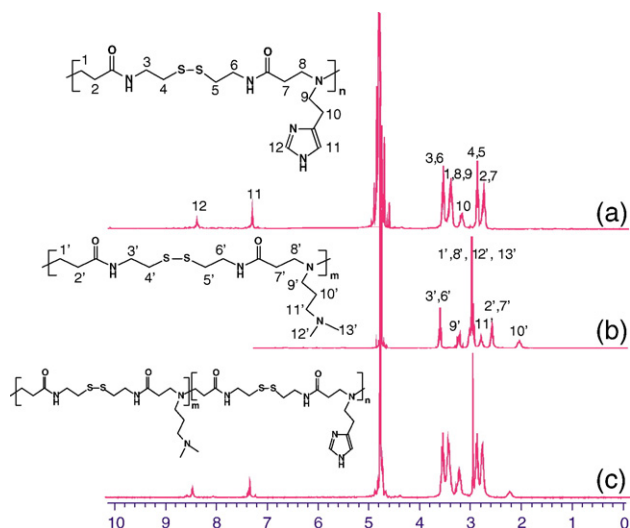
Fig. 1. ^{1}H NMR spectra (D$_2$O, 300 MHz) of (a) p(CBA-HIS) (Table 1, entry 1), (b) p(CBA-DMPA) (Table 1, entry 8) and (c) random p(CBA-HIS70/DMPA30) (Table 1, entry 2).

p(CBA-HIS) with terminal amino groups were synthesized at the appropriate HIS/DMPA ratio. Then, the two oligomers were mixed together and the reaction was continued for 2 days to yield the block copolymer. In a typical synthesis, taking the p(CBA-HIS70/DMPA30) block copolymer as an example, p(CBA-HIS) oligomer with average-number polymerization degree $\overline{X}_n = 19$ was prepared by stirring a mixture of CBA (0.72 g, 2.75 mmol) and excess of HIS (0.35 g, 3.06 mmol, stoichiometric ratio $r_{CBA/HIS} = 0.90$ mol/mol) in methanol/water (1.5 ml, 4/1 v/v) in a brown reaction flask at 45 °C in the dark under nitrogen atmosphere; meanwhile, p(CBA-DMPA) oligomer was prepared by adding DMPA (0.14 g, 1.31 mmol) and CBA (0.42 g, 1.61 mmol) in methanol/water (0.8 ml, 4/1 v/v) in another brown reaction flask. In this case, $r_{DMPA/CBA}$ is 0.81 to yield final molar equivalents of acrylamide and amino terminal groups (0.15 mmol) for the p(CBA-DMPA) oligomer and the p(CBA-HIS) oligomer. After 6 days of reaction, ^{1}H NMR spectra showed that the conversion of the acrylamide and amino terminal groups in the two reactions was more than 99%. Then, the two oligomer solutions were mixed together in a flask and allowed to react for 2 days. Subsequently, 10 mol % excess of DMPA (0.51 g, 0.44 mmol) was added to consume any unreacted acrylamide groups and stirring was continued for another 2 days at 45 °C. The resulting solution was diluted with water to about 30 ml, acidified with 6 M HCl to pH ~4, and then purified by ultrafiltration (5000 g/mol cut-off) with deionised water adjusted to pH ~4 with 0.1 M HCl. After freeze-drying the polymer in their HCl-salt form was collected as solid powder (0.7 g, 43%). The composition of these polymers was determined by ^{1}H NMR (D$_2$O, 300 MHz).

2.3. Polymer characterizations

^{1}H NMR spectra were recorded on Varian Inova spectrometer operating at 300 MHz.

The molecular weight and polydispersity (M_w/M_n) of the synthesized poly(amido amine)s were determined by GPC relative to PEO standards (Polymer Labs) using a Waters 2695 LC system (Milford, MA, USA) and two thermostated (30 °C) PL aquagel-OH 30 columns (8 μm, 300 × 7.5 mm, Polymer Labs, with a low-molar-mass separation range (200–40,000)). Data were collected using a differential refractometer (Model 2414). 0.3 M NaAc aqueous solution (pH 4.4) was used as eluent at a flow rate of 0.5 mL/min [21].

The buffering capacity of the SS-PAA copolymers was determined by acid–base titration as described previously [19]. An amount equal to 5 mmol of amine groups of the PAA copolymer was dissolved in 10 mL of 150 mM NaCl aqueous solution. The pH of the polymer solution was set at 2.0 and the solution was titrated with 0.1 M NaOH solution using an automatic titrator (Metrohm 702 SM Titrino). The buffering capacity defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.4, was calculated from equation:

$$\text{Buffer capacity } (\%) = \frac{\Delta V_{NaOH} \times 0.1M}{N mole} \times 100\%$$

wherein ΔV_{NaOH}, is the volume of NaOH solution (0.1 M) required to bring the pH value of the polymer solution from 5.1 to 7.4, and $N mole$, is the total moles of protonable amine groups in the PAA polymer (5 mmol).

2.4. Particle size and zeta-potential measurements

Polymer/plasmid DNA polyplexes at a polymer/DNA mass ratio of 12/1 (N/P ~ 16/1) were prepared by adding a HEPES buffer solution (20 mM, pH 7.4, 5 wt.% glucose) of polymer (800 μL, 225 μg/mL) to a HEPES buffer solution (20 mM, pH 7.4, 5 wt.% glucose) of plasmid DNA (200 μL, 75 μg/mL), followed by vortexing for 5 s and incubating at room temperature for 30 min. The surface charge and the size of polyplexes were measured at 25 °C with a Zetasizer 2000 instrument and a Zetasizer 4000, respectively (Malvern Instruments Ltd., Malvern, UK).

2.5. Agarose gel retardation

Polyplexes were made by adding 10 μL solution of SS-PAA copolymers or homopolymer mixtures (various concentrations in 20 mM pH 7.4 HEPES buffered saline (HBS)) to 10 μL plasmid solution (80 μg/mL in pH 7.4 HBS), followed by vortexing for 5 s and the dispersions were incubated for 30 min at room temperature. After addition of 5 μL of loading buffer containing bromophenol (Fermentas), 10 μL of this mixture was applied onto a 0.7% agarose gel containing 0.5 μg/mL ethidium bromide. After development of the gel, DNA was visualized with a UV lamp using a GelDoc system (Imago).

2.6. In vitro transfection and cell viability assays

Transfection experiments were performed with COS-7 cells (SV-40 transformed African Green monkey kidney cells) by using the plasmid pCMV-LacZ as reporter gene as reported previously [22,23]. Two parallel transfection series, one for the
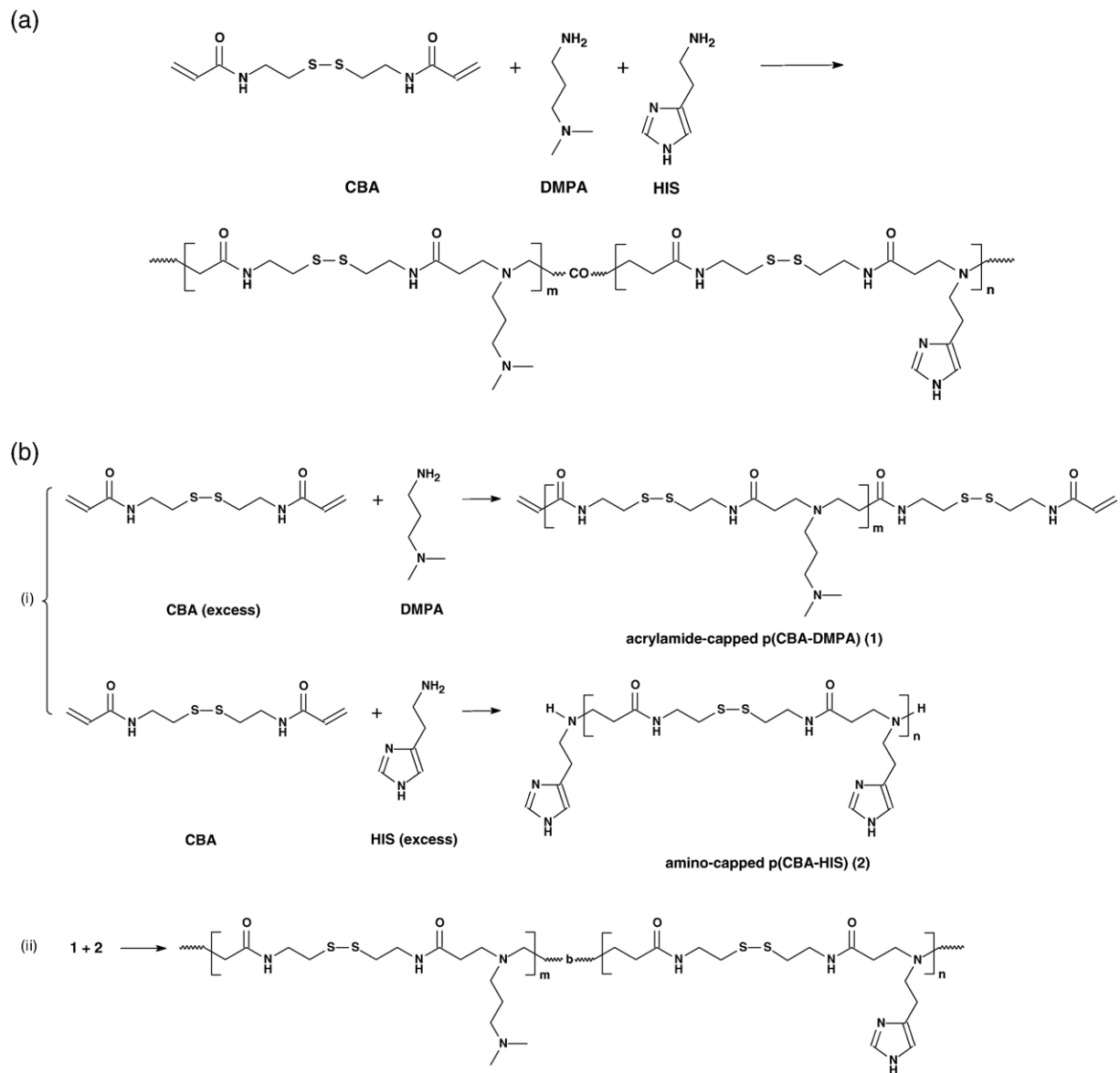
Scheme 1. Synthesis of bioreducible poly(amido amine) random (a) and block (b) copolymers.

determination of reporter gene expression (β-galactosidase) and the other for the evaluation of cell viability by XTT assay, were carried out in separate 96-well plates (ca. 1.0×10^4 cells per well). Different polymer/plasmid DNA weight ratios, ranging from 6 /1 to 24 /1 (w/w), were used to prepare the polyplexes. In brief, polyplexes were prepared by adding 200 μL of a HEPES buffer solution (20 mM, 130 mM NaCl, pH 7.4) of poly(amido amine) with varying concentrations (from 75 to 300 μg/mL) to 50 μL of a HEPES buffer solution (20 mM, 130 mM NaCl, pH 7.4) of plasmid DNA (50 μg/mL), followed by gentle shaking and incubating at room temperature for 30 min. The incubations of the polyplexes with the cells were performed either in the presence or absence of 5.0% serum. All transfection and toxicity assays were carried out in triplicate. In a standard transfection experiment, the cells were incubated with the desired amount of polyplexes (100 μL dispersion with 1 μg plasmid DNA per well) for 1 h at 37 °C in a humidified 5% CO_2-containing atmosphere.

Next, the polyplexes were removed. 100-μL of fresh culture medium was added and the cells were cultured for 2 days. The transfection efficiency was determined by measuring the activity of β-galactosidase using the ONPG assay [22]. A pEI/DNA formulation (Exgen 500) prepared at a nitrogen/phosphate (N/P) ratio of 10/1 was used as a reference. The number of viable cells was measured using an XTT assay [24]. The XTT value for untreated cells (i.e. cells not exposed to the transfection medium) was taken as 100% cell viability.

3. Results and discussion

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

Random copolymers of bioreducible poly(amido amine) (SS-PAA) with various ratios of histamine (HIS) and 3-

(dimethylamino)-1-propylamine (DMPA) were synthesized by Michael addition between N, N′-cystaminebisacrylamide (CBA) and a mixture of HIS and DMPA (Scheme 1a). The polymerization reactions were performed for 6 days by using molar equivalents of bisacrylamide (CBA) and amine monomer (HIS and DMPA). During the reaction time a gradual increase in viscosity was observed. To terminate the reaction, excess of DMPA was added to the reaction system and the reaction was continued for 2 days. Block SS-PAA copolymers with various HIS/DMPA ratios were obtained in a two-step process. First, the oligomers of acrylamide-terminated p(CBA-DMPA) and the oligomers of amino-terminated p(CBA-HIS) were synthesized. The number-average polymerisation degree ($\bar{X}_n$) of these oligomers was controlled by using a stoichiometric ratio (r) of the monomers (i.e., $r_{CBA/HIS}$ or $r_{DMPA/CBA}$) and was calculated with the equation: $\bar{X}_n = (1+r)/(1-r)$. Subsequently, these oligomers at various ratios of HIS/DMPA and molar equivalents of acrylamide and amino terminal groups were coupled by Michael addition reaction to form the respective block copolymers (Fig. 1b). These random and block SS-PAA copolymers were finally isolated as their HCl-salt by ultrafiltration and freeze-drying. They have a good solubility in water. The composition ratio of HIS and DMPA in the copolymer was determined by comparing the integrals of the ^{1}H NMR signals at δ 7.35 and δ 2.25, respectively, attributed to the proton (N=CH) in the HIS moiety and the methylene protons (CH$_2$CH_2CH$_2$) in the DMPA moiety (Fig. 1). The compositions of the copolymers are in accordance with the HIS/DMPA feed ratios (Table 1). In addition, for all polymers no signals were present between 5 and 7 ppm, corresponding to the acryl group, indicating that these copolymers are end-capped with amino groups. Gel permeation chromatography measurements showed that the weight-average molecular weight (M_w) of the SS-PAA polymers relative to PEO standard ranged from 5.8s k to 8.8 kg/mol with polydispersities (PDI = 1.25 ~ 1.88) (Table 1).

Table 1
Characterization of poly(amido amine) (PAA) homo- and copolymers

Entry	Poly(amido amine)	HIS/DMPA ratio [a]	M_w [b] (kDa)	M_w/M_n [b]	Buffer capacity [c] (%)
1	p(CBA-HIS)	–	7.6	1.53	58
2	Random p(CBA-HIS70/DMPA30)	66/34	6.3	1.65	51
3	Random p(CBA-HIS50/DMPA50)	52/48	6.1	1.88	42
4	Random p(CBA-HIS30/DMPA70)	29/71	5.8	1.45	38
5	Block p(CBA-HIS70/DMPA30)	72/28	7.4	1.25	50
6	Block p(CBA-HIS50/DMPA50)	52/48	8.5	1.34	41
7	Block p(CBA-HIS30/DMPA70)	32/68	8.8	1.60	38
8	p(CBA-DMPA)	–	8.7	1.41	32

[a] Determined by comparing the integrals of ^{1}H NMR signals at δ 7.35 and δ 2.25.
[b] Weight-average molecular weight (M_w) determined by GPC measurement.
[c] Buffer capacity of PAAs between pH 5.1 and 7.4 in 150 mM NaCl determined by acid–base titration.
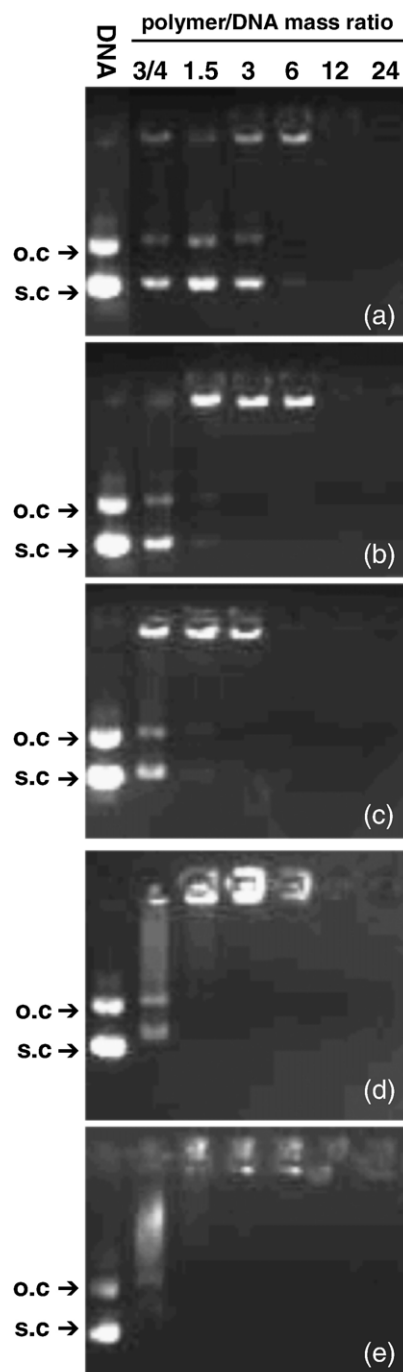

Fig. 2. Agarose gel electrophoresis of random PAA copolymer/pDNA polyplexes prepared at polymer/DNA mass ratios. (a) p(CBA-HIS), (b) p(CBA-HIS70/DMPA30), (c) p(CBA-HIS50/DMPA50), (d) p(CBA-HIS30/DMPA70) and (e) p(CBA-DMPA). (o.c.=open circular, s.c.=supercoiled form of plasmid DNA.)

3.2. Buffer capacity of bioreducible poly(amido amine) copolymers

The buffer capacity of the SS-PAA copolymers was determined from the acid–base titration curves ranging from pH 5.1 to pH 7.4. As is shown in Table 1, the nature of the amino groups in the side chain of the polymers has a distinct
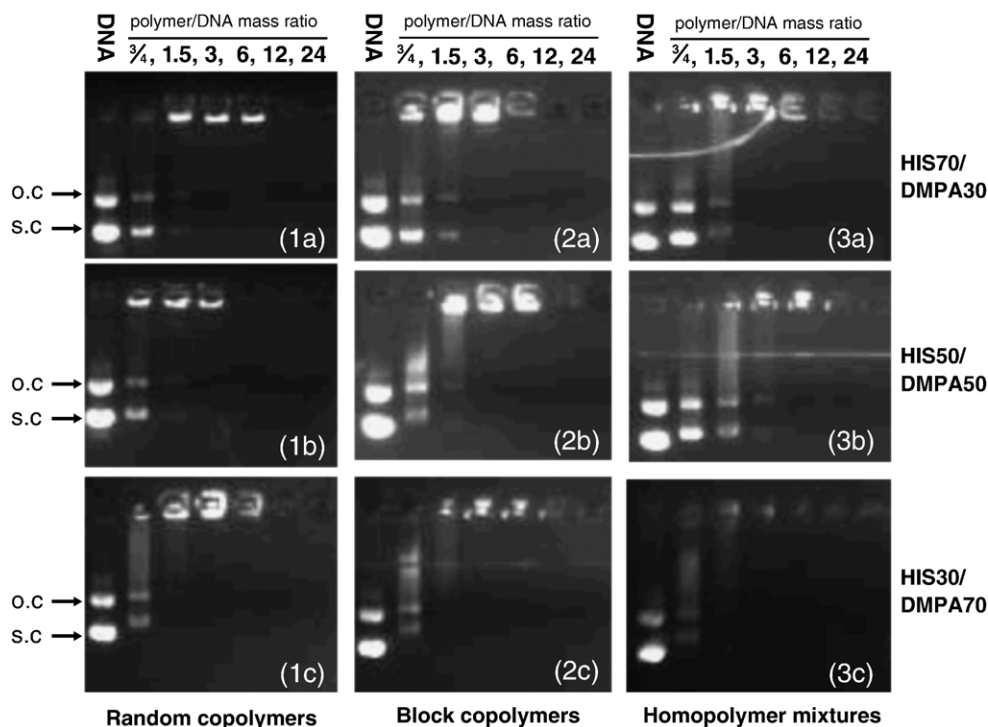
Fig. 3. Agarose gel electrophoresis of polyplexes based on HIS/DMPA random copolymers (1a, 1b, 1c), block copolymers (2a, 2b, 2c) and p(CBA-HIS)/p(CBA-DMPA) mixtures (3a, 3b, 3c) with various HIS/DMPA ratios of 70/30 (a series), 50/50 (b series) and 30/70 (c series) at various polymer/DNA mass ratios. (o.c.=open circular, s.c.=supercoiled form of plasmid DNA.)

effect on the buffer capacity. The homopolymer p(CBA-HIS) having only imidazole groups ($pK_a \sim 6.5$) in the side chain has a high buffer capacity of 58%, whereas the homopolymer p(CBA-DMPA) having only dimethyl amino groups ($pK_a \sim 8.0$) in the side chain shows a much lower buffer capacity of 32%. As can be expected from these results, the buffer capacities of both random and block copolymers decrease with decreasing HIS/DMPA ratio. No significant differences are observed between random and block copolymers at the same HIS/DMPA ratio. When the HIS/DMPA ratio decreases from 70/30 to 30/70 the buffer capacity decreases from 51% to 38%. This indicates that the buffer capacity of the copolymers can be nicely controlled by the HIS/DMPA ratio.

3.3. Characterization of DNA condensation of SS-PAA copolymers

Gel electrophoresis was performed to investigate binding behavior between copolymers and DNA. As is seen in Fig. 2, the homopolymer p(CBA-HIS) shows a relatively weak binding capability to DNA and total DNA retardation is detected at a relatively high polymer/DNA ratio of 12/1 (N/P ~ 16/1) (Fig. 2a). In contrast, the presence of DMPA into the copolymer has a clearly beneficial effect on the DNA binding capacity. All three random HIS/DMPA copolymers show good binding with DNA, similar to the homopolymer p(CBA-DMPA), at and above polymer/DNA mass ratios of 3/1 (N/P ~ 4/1) (Fig. 2b–e). Thus the incorporation of a low amount of DMPA into the copolymers is already sufficient to obtain copolymers with good DNA binding capacity.

Furthermore, the effect of structural differences of the PAA polymers on the DNA binding capacity was investigated by comparing polymer/DNA complexation of random copolymers,
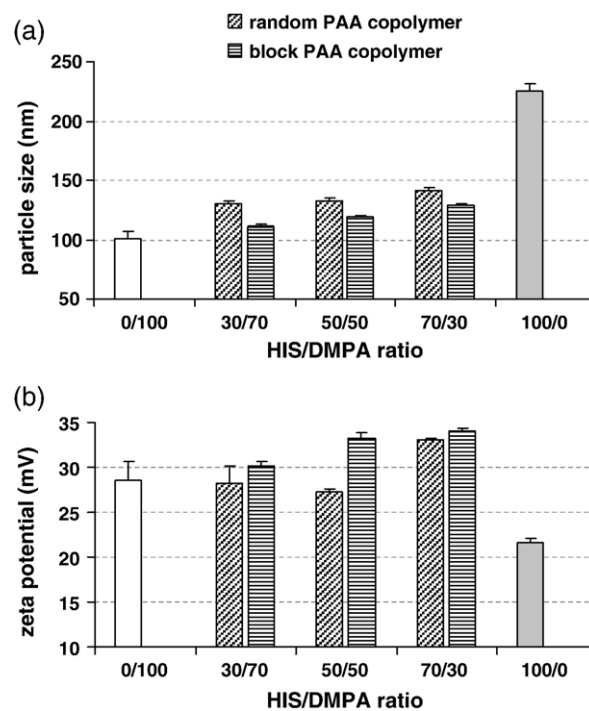

Fig. 4. Particle size (a) and zeta-potential (b) of polyplexes formed from random (upward diagonal) and block (horizontal) copolymers containing various HIS/DMPA ratios (30/70, 50/50 and 70/30), p(CBA-DMPA) (0/100) and p(CBA-HIS) (100/0) at a polymer/DNA mass ratio of 12/1 (N/P ~ 16/1). (20 mM HEPES, 5 wt.% glucose).
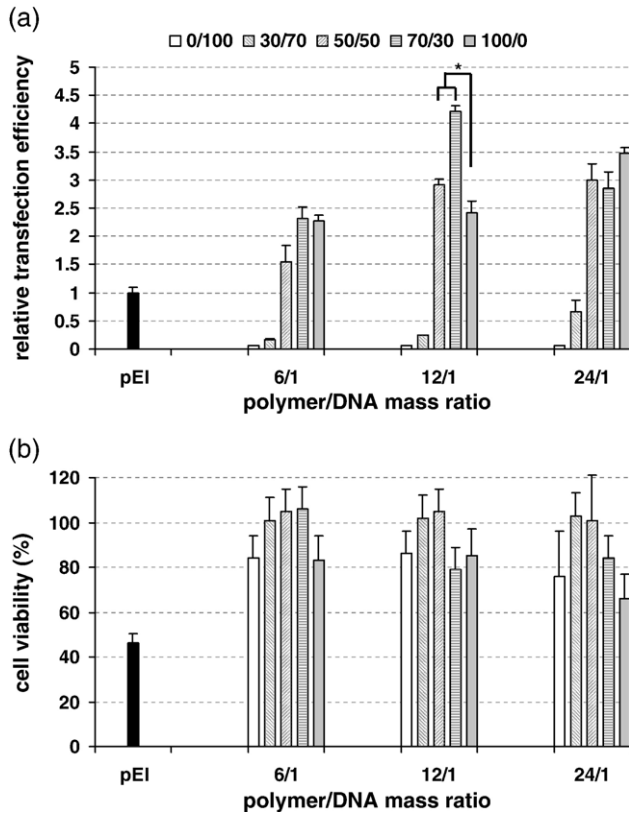
Fig. 5. Transfection efficiencies (a) and corresponding cell viabilities (b) of the polyplexes of random SS-PAA copolymers with varying HIS/DMPA ratios (30/70, 50/50 and 70/30, respectively), p(CBA-DMPA) (0/100) and p(CBA-HIS) (100/0) towards COS-7 cells at polymer/DNA mass ratios (6/1, 12/1 and 24/1). The transfection efficiency of pEI polyplexes at their optimal N/P ratio of 10/1 was taken as reference (set at 1.0). Cell viability was determined by the XTT assay. (Student's *t*-test, *$p < 0.05$).

block copolymers and mixtures of the homopolymers p(CBA-DMPA) and p(CBA-HIS) at the same HIS/DMPA ratios (70/30, 50/50 and 30/70, respectively). Total DNA retardation was observed for all three systems at and above polymer/DNA mass ratios of 3/1 (N/P ~ 4/1) and higher (Fig. 3). However, subtle differences in DNA binding were observed at a polymer/DNA mass ratio of 1.5/1 (N/P ~ 2/1), where random copolymers give slightly better DNA retardation than block copolymers, but both types of copolymers show stronger DNA binding than homopolymeric mixtures.

Fig. 4 shows the particle size and surface charge of polyplexes from random and block SS-PAA copolymers at a polymer/DNA mass ratio of 12/1 (N/P ~ 16), as determined by DLS and zeta-potential measurement, respectively. In general, block copolymers form polyplexes with a slightly smaller particle size and higher zeta-potential than random copolymers at the same HIS/DMPA ratios. Polyplexes from the homopolymer p(CBA-HIS) have relatively the largest size (~ 210 nm) and the size of the polyplexes decreases with increasing ratio of DMPA/HIS in the copolymers (~ 110–140 nm) (Fig. 4a). Polyplexes of p(CBA-DMPA) and p(CBA-HIS/DMPA) copolymers have higher positive zeta-potentials (27 ~ 33 mV) than those of p(CBA-HIS) (~ 22 mV). Notably, polyplexes of

random and block p(CBA-HIS70/DMPA30) copolymers show even higher zeta-potentials (~ 32–33 mV) than those of p(CBA-DMPA) (~ 29 mV) (Fig. 4b).

3.4. In vitro transfection efficiency and cytotoxicity

The transfection of polyplexes based on the SS-PAA polymers was studied *in vitro* by using COS-7 cells and the plasmid pCMV-LacZ as reporter gene and their cell viabilities were evaluated by XTT assays. First the effect of polymer/DNA ratio was evaluated by measuring the transfection efficiency at polymer/DNA mass ratios of 6/1, 12/1 and 24/1 (N/P ~ 8, 16 and 32), respectively. Fig. 5a shows that normalized to that of ExGen 500 (linear pEI, 22 kDa), polyplexes containing 50% or higher ratio of HIS (co) polymer all give transfection efficiencies that are significantly higher than that of the reference polymer pEI. Moreover, optimal transfection efficiency of the random copolymers is obtained for the copolymer with HIS/DMPA ratio of 70/30 at a polymer/DNA mass ratio of 12/1. Generally, the random copolymers show an improved transfection efficiency with increasing HIS content, which could be correlated with the increased buffer capacity of these copolymers (Table 1). Notably, at the ratio of 12/1 polyplexes of p(CBA-HIS50/DMPA50) and p(CBA-HIS70/DMPA30) give
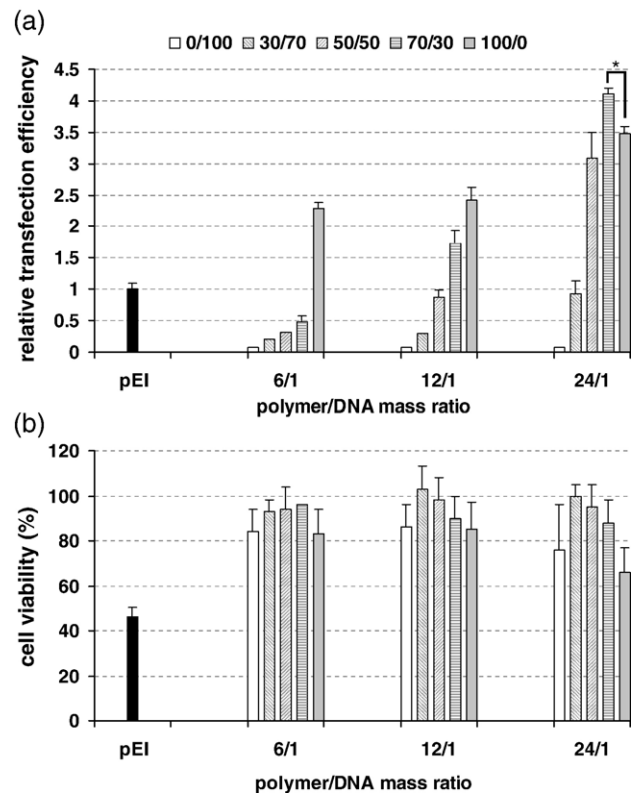

Fig. 6. Transfection efficiencies (a) and corresponding cell viabilities (b) of the polyplexes of block SS-PAA copolymers with varying HIS/DMPA ratios (30/70, 50/50 and 70/30, respectively), p(CBA-DMPA) (0/100) and p(CBA-HIS) (100/0) towards COS-7 cells at polymer/DNA mass ratios (6/1, 12/1 and 24/1). The transfection efficiency of pEI polyplexes at their optimal N/P ratio of 10/1 was taken as reference (set at 1.0). Cell viability was determined by the XTT assay. (Student's *t*-test, *$p < 0.05$).
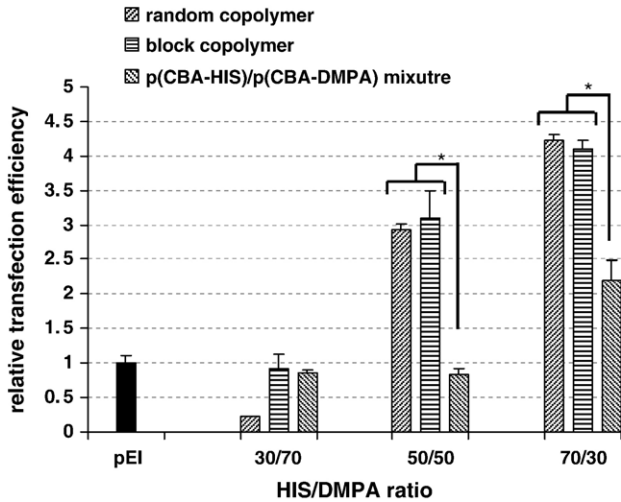
Fig. 7. Transfection efficiencies of the polyplexes of random copolymers (upward diagonal), block copolymers (horizontal) and p(CBA-HIS)/p(CBA-DMPA) mixture (downward diagonal) with various HIS/DMPA ratios towards COS-7 cells at their optimized polymer/DNA mass ratios of 12/1, 24/1 and 24/1, respectively in the absence serum. The transfection efficiency of pEI polyplexes at their optimal N/P ratio of 10/1 was taken as reference (set at 1.0). (Student's t-test, $*p < 0.05$).

higher transfection than p(CBA-HIS) based polyplexes. This improved efficiency may be related to the smaller particle size and higher surface charge of the copolymer based polyplexes (Fig. 4a and b). The cytotoxicity profiles of the polyplexes are shown in Fig. 5b. Compared to pEI, which shows a cell viability of only 40%, the polyplexes of all p(CBA-HIS/DMPA) (co)polymers show essentially low or absence of cytotoxicity with cell viability of 80% and higher at all polymer/DNA ratios. From our earlier work [18,19], it can be concluded that the presence of the bioreducible disulfide bonds in the main chain of these poly(amido amine)s favorably contribute to the low cytotoxicity as the polymers can be rapidly degraded by disulfide cleavage by glutathione and reductase enzymes in the cellular interior.

The polyplexes of the block copolymers show transfection profiles similar to those of the random copolymers. Their transfection efficiencies increase with increasing HIS/DMPA ratio (Fig. 6a). However, a higher polymer/DNA ratio is required for optimal transfection efficiency as compared to that of random copolymers (24/1 vs. 12/1). Polyplexes of block p(CBA-HIS70/DMPA30) copolymer at a polymer/DNA ratio of 24/1 give ca. 1.2 times higher transfection than those of p(CBA-HIS) and ca. 4.1 times more effective than pEI. Also, the polyplexes of the block copolymers display essentially low cytotoxicity with cell viabilities of 90% at polymer/DNA ratios where optimal transfection is observed (Fig. 6b).

The effect of the polymer structure and formulation on the transfection of polymers, i.e. the presence of random copolymer, block copolymer or a mixture of p(CBA-HIS) and p(CBA-DMPA) is shown in Fig. 7. The polyplexes of random and block copolymers at their optimal polymer/DNA mass ratios induce similar transfection efficiency, indicating that the structural pattern in the polymer (random vs. block) has only little effect. However, polyplexes from both copolymers induce significant-

ly higher efficiency than polyplexes composed of p(CBA-HIS)/p(CBA-DMPA) mixtures. This may serve as an indication that functionalities that predominantly contribute to favorable gene delivery properties as DNA condensation (DMPA) and buffer capacity (HIS) are more effectively combined by intimate covalent assemblation than by (loose) physical mixtures. This conclusion is in line with recent results of Kataoka et al., who reported that pLL-based block copolymers showed more efficient transfection towards HeLa cells than a physical blend of two pLL-based homopolymers [15].

It is well known that the presence of serum often causes aggregation of polyplexes, resulting in decreased transfection efficiency [25]. The transfection efficiencies of the copolymers in the presence of serum at their optimal polymer/DNA ratio are shown in Fig. 8a. The polyplexes of random and block copolymers at HIS/DMPA ratio of 70/30 again induce the highest transfection efficiencies along with cell viability >90% (Fig. 8b). Also here, the transfection efficiency of the copolymers is much higher than that of p(CBA-HIS)/p(CBA-DMPA) mixtures. Compared to the serum-free medium, the presence of 5% serum
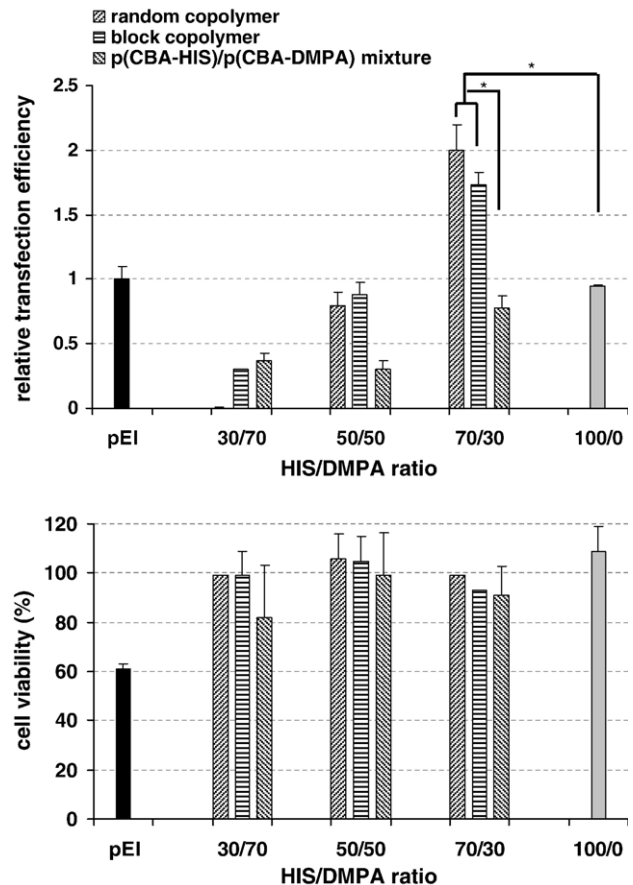

Fig. 8. Transfection efficiencies (a) and corresponding cell viability (b) of the polyplexes of random copolymers (upward diagonal), block copolymers (horizontal) and p(CBA-HIS)/p(CBA-DMPA) mixture (downward diagonal) with various HIS/DMPA ratios towards COS-7 cells at their optimized polymer/DNA mass ratios of 12/1, 24/1 and 24/1, respectively in the presence of 5% serum. The transfection efficiency of pEI polyplexes at their optimal N/P ratio of 10/1 was taken as reference (set at 1.0). Cell viability was determined by the XTT assay. (Student's t-test, $*p < 0.05$).

decreases the transfection efficiency of the polyplexes of p(CBA-HIS70/DMPA30) by about 2 times and this decrease is relatively lower than that observed for p(CBA-HIS).

4. Conclusions

We have demonstrated that bioreducible poly(amido amine) (SS-PAA) with tunable DNA condensation capacity and buffer capacity can be readily obtained by synthesis of random and block copolymers with controlled composition from Michael addition polymerization between cystaminebisacrylamide (CBA) and two amine monomers, *i.e.*, histamine (HIS) and 3-(dimethylamino)-1-propylamine (DMPA). The presence of a low percentage of DMPA into the p(CBA-HIS/DMPA) copolymer already results in an improved DNA binding behavior that is comparable to the p(CBA-DMPA) homopolymer and is significantly higher than the p(CBA-HIS) homopolymer. The buffer capacities of the copolymers can be controlled by the amino side group composition of the copolymer and increases with increasing HIS/DMPA ratio. The SS-PAA copolymers are capable to transfect COS-7 cells *in vitro* with efficiencies that increase with increasing HIS/DMPA ratio. The random and block copolymers with a HIS/DMPA ratio of 70/30 combine high DNA condensation ability and buffer capacity, thereby inducing higher transfection efficiency than p(CBA-HIS) homopolymer in the absence and presence of serum. Moreover, the serum has only a small influence on the transfection efficiency of polyplexes of the copolymer as compared to those of the homopolymer. Differences in polymeric structure, i.e. a random or a block pattern, have only marginal effect on transfection capacity of copolymers, but covalent assemblage of the HIS and DMPA functional groups in a single polymer chain clearly results in better transfection properties than the use of mixtures of p(CBA-HIS) and p(CBA-DMPA) homopolymers in the polyplexes. Polyplexes formed from the SS-PAA random and block copolymers reveal much higher transfection efficiency and lower cytotoxicity than those of pEI, indicating they have significant potential for application as safe and efficient gene delivery vectors.

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