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Poly(Amido Amine)s Containing Agmatine and Butanol Side Chains as Efficient Gene Carriers

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Abstract

A new type of bio reducible poly(amido amine) copolymer is synthesized by the Michael addition polymerization of cystamine bisacrylamide (CBA) with 4-aminobutylguanidine (agmatine, AGM) and 4-aminobutanol (ABOL). Since the positively charged guanidinium groups of AGM and the hydroxybutyl groups of ABOL in the side chains have shown to improve the overall transfection efficiency of poly(amido amine)s, it is hypothesized that poly(CBA-ABOL/AGM) synthesized at the optimal ratio of both components would result in high transfection efficiency and minimal toxicity. In this study, a series of the poly(CBA-ABOL/AGM) copolymers is synthesized as gene carriers. The polymers are characterized and luciferase transfection efficiencies of the polymers in various cell lines are investigated to select the ideal ratio between AGM and ABOL. The poly(CBA-ABOL/AGM) containing 80% AGM and 20% ABOL has shown the best transfection efficiency with the lowest cytotoxicity, indicating that this polymer is very promising as a potent and nontoxic gene carrier.

Graphical abstract

Keywords

biological applications of polymers; drug delivery systems; gene delivery; nanoparticles; structure-property relations; transfection

1. Introduction

Gene therapy requires the transfer of a therapeutic gene to target cells with high efficiency. A key limitation to the development of human gene therapy is the lack of safe, efficient, and controllable methods for gene delivery. ^[1–3] Gene delivery vehicles can be divided into two categories: viral vectors and nonviral polymer carriers. Viral vectors have high transfection efficiencies, but their use is limited by their inherent immunogenicity and their potential for integration into the host genome. ^[4–6] The development of nonviral carriers has typically been based on three principles: electrostatic interaction, encapsulation, and absorption. ^[7–9] Polyplexes formed by the electrostatic interaction of cationic polymers with nucleic acids appear to have an excess charge density to facilitate binding to the cell membrane and internalization into cells followed by transfection. ^[10,11] However, the high charge density often causes increased toxicity. Reducible cationic polymers have been known to have a high enough charge density to condense DNA or siRNA and minimize the toxicity associated with the excess positive charge through the reduction of the polymer into its counter monomers inside cells. ^[12,13]

Among various types of poly(amido amine)s (PAA)s, the most studied reducible polymer poly(CBA-4-aminobutanol), obtained by the Michael-type polyaddition of 4-aminobutanol (ABOL) to cystamine bisacrylamide (CBA) showed remarkably efficient transfection efficiency. ^[14] This performance was correlated to a favorable contribution of the butanol side chains to the interaction of the copolymer with the cell membrane through hydrophobic interaction ^[14,15] and the high proton uptake capacity (buffer capacity) of this polymer in the range of endosomal acidification, with pK_a values around 6 of the tertiary nitrogens in the main chain of the poly(CBA-ABOL) polymer. However, the stability of this polyplex is relatively weak, due to limited protonation of these tertiary nitrogens at neutral pH. In contrast, poly(CBA-AGM), the polymer obtained from CBA with 4-aminobutylguanidine (agmatine, AGM), possesses high cationic charge due to complete protonation of the guanidine groups at neutral pH, resulting in efficient and stable polyplex formation and cell adhesion by electrostatic interactions. ^[16–19] It is well known that the guanidinium groups in cell penetrating peptides play a critical role in the binding with nucleic acids and gene packing followed by gene transfection. ^[4,20] In a previous study it was shown that introduction of guanidinium groups into a PAA polymer exhibited gene transfection efficiency that was in direct proportion to the ratio of the guanidinium group added. ^[4]

The objective of this study was to develop a new type of PAA containing both AGM and ABOL, which can together promote polyplex formation, cell binding, cellular uptake, endosomal escape, and intracellular release of DNA or siRNA by the cleavage of disulfide bonds, which in turn results in enhanced transfection. The overall net charge of the PAA containing AGM depends on the content of AGM within the copolymer because the guanidine group of AGM is the primary source of the cationic charge. PAA polymerized with AGM has the advantage of high charge density with negligible toxicity to cells and strong proton buffering capacity, both of which make AGM-containing PAAs unique as gene carriers. [21–23]

We prepared five types of the poly(CBA-ABOL/AGM) copolymer that contain different molar ratios of AGM and ABOL to CBA and evaluated the transfection efficiencies of those copolymers. The optimum ratio between AGM and ABOL was chosen based on the transfection efficiencies and toxicities of the series of copolymers. Polyplexes from poly(CBA-ABOL/AGM) prepared at 80% AGM and 20% ABOL showed better transfection efficiency than those of PEI in various types of cells, together with low toxicity.

2. Experimental Section

2.1. Materials

Acryloyl chloride (99.8%), DMSO- d_6 (99.9%), deuteriumoxide (99.9%), deuterium chloride (DCI: 99.9%), and 20% HCl in D₂O were obtained from Merck (Darmstadt, Germany). Cystamine dihydrochloride (>96%), lithium hydroxide monohydrate (>99%), 4-amino-1-butanol (>98%), sodium hydroxide (>97%), and agmatine sulfate (>97%), poly(ethylenimine) (PEI; branched form, $M_w = 25$ kDa), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO). Ultrapure water was obtained from a Synergy-2 Millipore apparatus. All cell culture products including fetal bovine serum, Dulbecco's phosphate buffered saline, and Dulbecco's modified Eagle's medium were obtained from Invitrogen (GibcoBRL; Carlsbad, CA). The Luciferase assay system and reporter lysis buffer were from Promega (Madison, WI).

2.2. Synthesis of *N,N'*-Cystamine-bis-Acrylamide

N,N'-cystamine-bis-acrylamide (CBA) was synthesized from cystamine dihydrochloride and acryloyl chloride according to the following standard procedure: Cystamine dihydrochloride (50.0 g; 0.22 mole) was added to 120 mL sodium hydroxide solution (8.3 M; 1.0 mole) in a three-neck round bottomed flask and kept stirred until dissolved. While cooling at 0 °C, 65 mL acryloyl chloride (10.2 M; 0.67 mole) in dichloromethane was added while stirring vigorously. After 1 h of stirring the temperature was raised to the room temperature and the reaction was continued overnight. Dichloromethane was removed by decanting and the crude CBA was collected by filtration. The crude CBA was washed with 4 × 50 mL ultrapure water and then recrystallized twice from 250 mL ethyl acetate followed by washing with 3 × 30 mL cold ethyl acetate. Pure CBA was vacuum-dried and analyzed by nuclear magnetic resonance (NMR).

2.3. Synthesis of a Series of Poly(CBA-AGM_x/ABOL_y)

A series of poly(CBA-AGM_x/ABOL_y) was synthesized by Michael addition according to the following standard procedure. One gram [3.84 mmole] of CBA was dissolved in 5 mL of methanol. One equivalent of the combined amine monomers and 1 equivalent of lithium hydroxide monohydrate, to agmatine sulfate, were dissolved in 5 mL ultrapure water. The CBA solution was added to the water solution and the mixture was stirred for 12 d at room temperature, shielded from light. Polymerizations with agmatine content >80% may start to precipitate during the reaction and need further diluting with methanol. The polymerization was quenched, at an acryl consumption >92% as detected by NMR, by endcapping with the amine monomers. Therefore, the pH was set at 9 and 25 mol% excess of the appropriate ratio of the amine monomers was added to the polymer solution which was further stirred for 8 d at the room temperature under protecting from light. The pH was subsequently raised to 4 and the polymer solutions were purified by dialysis with ultrapure water, using a cutoff filter of 1 kDa. The polymers were isolated by lyophilization and kept at -80 °C until use.

2.4. Molecular Weight Determination

Molecular weights of the series of poly(CBA-ABOL_x/AGM_y) were determined by GPC (Waters; e2695) equipped with a column, Aquagel-OH 30 8 μm (300 × 7.5 mm). The polymer samples were run in 0.3 M sodium acetate buffer, pH = 4, and 20% methanol. Maldi-Tof was further performed using a Waters-Maldi Synapt, High Definition Mass Spectrometer. All samples were measured using 2,5-dihydroxy benzoic acid and α-cyano-4-hydroxy cinnamic acid matrixes.

2.5. NMR

All measurements were done using D₂O/DCI and DMSO-*d*₆ using a Bruker 400 MHz NMR, CR 4315. The best result was obtained with D₂O/DCI. The increasing/decreasing peak intensity of the methylene protons of ABOL next to the hydroxyl group corresponds to its feed ratio used for the polymerization. ¹H-NMR (D₂O/DCI, 300 MHz): -SCH₂ (2.82–2.92 ppm), -SCH₂CH₂ (3.47–3.56 ppm), -NHCOCH₂ (2.72–2.82 ppm), -NHCOCH₂CH₂ (3.18–3.28 ppm), -NCH₂ (3.38–3.56 ppm), -NCH₂CH₂ (1.75–1.90 ppm), -NCH₂CH₂CH₂ [1.55–1.72 ppm], -CH₂OH [3.57–3.64 ppm], -CH₂NC(NH₂)₂ [3.38–3.56 ppm]. Some observed smaller peaks can be contributed to methylene groups adjacent to a low fraction of unprotonated tertiary the amine groups.

2.6. Gel Retardation

Poly (CBA-ABOL/AGM) dissolved in PBS was mixed with 1 μg pSV-Luci at various weight ratios. The mixtures were incubated for 20 min at room temperature and electrophoresed on 1% (w/v) agarose gel for 30 min at 100 V in 0.5% TBE buffer solution. Ratios were calculated as weight ratios of the polymer to the pSV-Luci for all data. To investigate the reducibility of the polyplex, the poly (CBA-ABOL/AGM)/Luci polyplex prepared at the optimal weight ratio was incubated in the presence of various concentrations of DTT. The samples were reacted under gentle stirring for 2 h at 37 °C and then electrophoresed as described above.

2.7. Polyplex Characterization

Mean diameters and zeta potentials of the poly (CBA-ABOL₂₀/AGM₈₀)/Luci or the poly (CBA-AGM₁₀₀)/Luci polyplex prepared at 3/1 or 5/1 weight ratios were measured using dynamic light scattering (Zetasizer-Nano ZS; Malvern Instruments, Worcester-shire, UK). Four micrograms of the pSV-Luci were mixed with one of the polymers at weight ratios of 3/1 or 5/1 (polymer/DNA) in 1 mL PBS (pH 7.4).

2.8. In Vitro Transfection

Human embryonic kidney 293 (HEK 293) cells, mesenchymal stem cells (MSC)s, or A2780 human ovarian carcinoma cells were seeded on 24-well plates at a density of 2.0×10^4 cells per well when cell confluence reached 80%. After 24 h postseeding, the culture media were replaced with plain media containing the poly (CBA-ABOL/AGM)/pSV-Luci polyplexes prepared by mixing 1 μ g pSV-Luci with different amounts of the polymer or 1 μ g PEI. The culture media were replaced with the complete media after 4 h of incubation. At 48 h post-transfection, cells were washed with PBS and lysed with 100 μ L reporter lysis buffer according to the manufacturer's instructions. The cells were harvested and centrifuged for 30 s at 13 000 rpm. Luciferase RLU of the cells was measured using a 96-well plate luminometer, and the results were expressed as RLU per mg protein determined by a micro BCA assay kit with a BSA standard. Cell viabilities were determined by MTT assay. In addition, the cells were transfected with polyplexes in the presence of 10% serum without changing the media. Luciferase activity was determined 48 h after the transfection as detailed above.

3. Results and Discussion

3.1. Synthesis of the Series of Poly(CBA-ABOL/AGM)s

To combine the advantages of both AGM and butanol derivatives in regard to transfection efficiency, a series of copolymers, poly(CBA-ABOL/AGM)s, having different contents of ABOL and AGM was prepared by reacting an equimolar amount of CBA with an equimolar amount of a mixture of ABOL and AGM at one of the following ABOL:AGM ratios; 0:100 (AGM100), 20:80 (AGM80), 40:60 (AGM60), 80:20 (AGM20), or 100:0 (AGM0) (Figure 1). The purified copolymers were obtained with an average yield of 25–50 wt% and molecular weights of AGM100 = 1010, AGM80 = 964, AGM60 = 984, AGM20 = 976, and AGM0 = 981 (g mole^{-1}) determined by GPC. The maximum molecular weights of the polymers obtained as measured with mass spectrometry are AGM100 = 2864, AGM80 = 2302, AGM60 = 2260, AGM20 = 2528, and AGM0 = 2448 (g mole^{-1}), respectively. For the copolymers basically all possible monomer combinations, up to the max M_w , can be found in the mass spectrum. The ratios of constituting monomers corresponded with the feed ratios used.

3.2. Characteristics of the Poly(CBA-ABOL_x/AGM_y)/DNA Polyplexes

AGM and ABOL were copolymerized with CBA via the Michael poly-addition reaction to yield the poly(CBA-ABOL_x/AGM_y). These reducible PAA copolymers can fragment via the reduction of the disulfide bonds present within the copolymer in the presence of reducing

agents or in the reducing environment of the cytosolic space and cell surface. [24] Cationic polymers including poly(CBA-ABOL/AGM) as gene carriers have to be capable of assembling DNA into nanosized polyplexes and this complexation occurs through electrostatic interaction. The charge density of the poly(CBA-ABOL/AGM) copolymer at neutral pH is primarily attributed to the protonated AGM side chains. The ABOL side groups function as an enhancer for the interaction with the cell membrane to increase the internalization of the polyplex into the cells. Since the charge of the poly(CBA-ABOL/AGM) is expected to increase with increasing amount of AGM in the copolymer, AGM80 and AGM100 appear to have a greater ability to condense DNA. Gel retardation assays conducted to evaluate the capabilities of the copolymers for DNA condensation show that DNA migration was completely retarded at the following weight ratios (w/w polymer/DNA); AGM0: 10 w/w, AGM20: 5 w/w, AGM60: 3 w/w, AGM80: 2 w/w, and AGM100: 2 w/w (Figure 2). There was no significant difference in DNA condensation capacity among AGM60, AGM80, and AGM100, while AGM0 and AGM20 condensed DNA only at relatively high weight ratios. As AGM80 and AGM100 turned out to have stronger DNA condensation ability superior to the other copolymers, we determined the mean diameter and the zeta potential of the AGM80/DNA or AGM100/DNA polyplexes. The AGM80/DNA polyplex prepared at a weight ratio of 3 had a size of 70.1 ± 1.0 nm with a PDI value of 0.324 ± 0.015 , and a zeta potential of $+31.0 \pm 1.1$ mV. The AGM100/DNA polyplex prepared at 3 w/w had a size of 73.5 ± 2.0 nm with a PDI value of 0.326 ± 0.041 and a zeta potential of $+30.5 \pm 0.3$ mV. It has been widely agreed that sizes for polyplexes required for the internalization into cells and the subsequent processing in the cells should be ideally less than 400 nm in diameter. [25] The physicochemical features of the poly(CBA-ABOL/AGM)/DNA polyplex make this copolymer effective to transfect DNA in terms of the size, surface charge, and biodegradability by reductive disulfide cleavage.

The disulfide linkages in the PAAs are stable in the extracellular environment but are sensitive for cleavage in the reducing environment of the cytosol, thereby initiating the dissociation of the PAA/DNA polyplexes. Glutathione plays a major role in this cleavage by the disulfide exchange reaction and it is known that the glutathione contents is in the cytosol about 1000-fold higher than in the extracellular milieu. [26] This implies that the levels of glutathione (e.g., ranging from 0.5 to 11×10^{-3} M in liver cells) [27] play a critical role in triggering the release of DNA upon internalization of the PAA/DNA polyplex, which in turn facilitates the gene expression. The poly(CBA-ABOL/AGM) is an example of a reducible PAA prone to responding to glutathione. Therefore, the behavior of the AGM80/DNA polyplex and the AGM100/DNA polyplex was investigated in the presence of the reducing agent DTT (as a model reagent for glutathione) by a gel retardation assay. As shown in Figure 3, the polyplexes dissociated when incubated with increasing concentrations of DTT and free DNA was released from the polymers, whereas DNA persisted in a condensed form by the polymer in the absence of DTT. This indicates that the AGM80 copolymer and the AGM100 copolymer can be degraded by reductive cleavage of the disulfide bonds in the PAA. In the reducing environment of the cytosol degradation of the cationic polymer will result in release of the condensed DNA, which in turn facilitates gene expression and decrease of cytotoxicity that is generally caused by polycations. [28–30]

3.3. In Vitro Transfection

The five types of the poly(CBA-ABOL/AGM) copolymers are expected to have different transfection efficiencies depending on the AGM content. We compared the transfection efficiencies of the five types of the copolymers in three different types of cells, HEK293, MSC, and A2780 because each cell has different levels of glutathione, indicating that the transfection efficiencies of the poly(CBA-ABOL/AGM) need to be determined in varying types of cells. Since each of the polymers could condense DNA at varying weight ratios depending on the amounts of AGM contained, poly(CBA-ABOL/AGM)/DNA polyplexes were prepared for transfection by mixing a fixed amount of DNA with different amounts of each copolymer based on the results obtained from the gel retardation assays. The transfection efficiencies of the copolymers were first examined in a ratio-dependent manner in the absence of serum in order to find which of the copolymers is the most effective in gene transfection. The transfection efficiencies of AGM0 and AGM20 were lower than that of PEI in HEK293 cells (Figure 4) and MSCs (Figure 5), whereas AGM60, AGM80, and AGM100 had higher levels of luciferase expression in those cells (Figures 4 and 5). In A2780 cells, all of the copolymers showed better transfection efficiencies than PEI (Figure 6). Compared to PEI, AGM0 and AGM20 showed almost the same levels of transfection in HEK293 cells at the ratios of 40 and 10, respectively, whereas they could not lead to the luciferase expression in MSCs and higher gene expression than PEI in A2780 cells. However, AGM60, AGM80, and AGM100 exhibited higher gene expression than PEI in all three-cell lines when complexed with DNA at appropriate weight ratios. The luciferase activities were increased in all three cell lines tested in a polymer ratio-dependent manner. The difference between AGM0 and AGM100 is mainly due to the content of AGM in the final copolymer. Figures 4–6 showing the effect of the AGM content on transfection efficiency reveal that AGM80 and AGM100 are the most effective copolymers among the five types of copolymers in terms of their transfection efficiencies and the minimum ratio required for a reasonable gene expression. In this series of the poly(CBA-ABOL/AGM) copolymers, it is possible to note that there is a direct proportion between the contents of AGM contained within the copolymer and the transfection efficiency.

Based on these results, we chose AGM80 and AGM100 for transfection in the presence of serum because the polyplexes may aggregate in the presence of serum, resulting in a decrease in the levels of gene transfection. [20] The transfection efficiencies of PEI, AGM80, and AGM100 decreased in the presence of 20% serum by ≈ 5 times. Whereas PEI decreased the cell viability to less than 50% in both cell lines, the poly(CBA-ABOL/AGM) copolymers are relatively nontoxic, illustrating a superior qualities when compared to PEI. Although AGM100 shows the highest transfection efficiency, AGM80 appeared to be $\approx 5\%$ – 20% less cytotoxic, depending on the type of cells when compared to AGM100. Although there are negligible differences between AGM80 and AGM100, AGM80 is accepted as the most promising gene carrier among the five types of the poly(CBA-ABOL/AGM) copolymers with regard to its higher transfection efficiency and lower cytotoxicity.

4. Conclusion

We developed a new family of PAA copolymers as gene carriers by polymerization of ABOL and AGM on the CBA backbone, which results in the formation of the poly(CBA-ABOL/AGM) copolymer, which is nontoxic to cells and effective for gene transfection. We have verified that AGM80, which contains 80% AGM and 20% ABOL, is the best poly(CBA-ABOL/AGM) copolymer for gene transfection among the copolymers tested in this study. This would be due to the fact that AGM80 contains the advantages of AGM side chains providing a high DNA condensing capacity and binding to the cell membrane, tertiary amines for endosomal buffering, and ABOL side groups enabling aided cell membrane interactions. Conclusively, this new class of PAAs has key features as a potent and safe gene carrier. Moreover, the ease of synthesis and the possibility of modification offer an opportunity to further develop a next generation of the poly(CBA-ABOL/AGM) copolymer containing other functional domains. This allows altering the biophysical properties of the polyplexes or adding targeting ligands by affixing homing peptides, which in turn improves in vivo gene delivery. The poly(CBA-ABOL/AGM) synthesized by the Michael polyaddition of AGM and ABOL to CBA is a very promising nonviral gene carrier in regards to the gene expression and required low toxicity.

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Figure 1.
Chemical structure of the poly(CBA-ABOL/AGM) copolymer.

Figure 2. Gel retardation assay. DNA was incubated with five types of the poly(CBA-ABOL/AGM) copolymers and PEI at different weight ratios for 20 min in PBS (pH 7.4). The numbers indicate the weight ratios of the polymer/DNA.

Figure 3. Polyplex dissociation in the presence of DTT. AGM80/DNA or AGM100/DNA polyplexes were incubated with increasing concentrations of DTT for 2 h. The numbers indicate DTT concentration in mM.

Figure 4. Levels of luciferase activity in HEK293 cells. Transfection efficiencies of a) AGM0, b) AGM20, c), AGM60, d) AGM80, and e) AGM100 were determined and compared with that of PEI. Luciferase activity was measured 48 h after the transfection. The numbers in the figure legend indicate weight ratio of the copolymer/DNA.

Figure 5. Levels of luciferase activity in MSCs. Transfection efficiencies of a) AGM0, b) AGM20, c), AGM60, d) AGM80, and e) AGM100 were determined and compared with that of PEI. Luciferase activity was measured 48 h after the transfection. The numbers in the figure legend indicate weight ratio of the copolymer/DNA.

Figure 6. Levels of luciferase activity in A2780 cells. Transfection efficiencies of a) AGM0, b) AGM60, c), AGM80, and d) AGM100 were determined and compared with that of PEI. Luciferase activity was measured 48 h after the transfection. The numbers in the figure legend indicate weight ratio of the copolymer/DNA.

Figure 7. Transfection efficiencies of AGM80 and AGM100 in HEK293 cells or MSCs in the presence of serum. Transfection efficiencies of AGM80 and AGM100 were evaluated in the presence of serum in a) HEK293 cells and b) MSCs. Luciferase activity was measured 48 h after the transfection. The polyplexes were prepared at the weight ratio of 5.

Figure 8. Cell viability. Toxicities of a,c) AGM80 and b,d) AGM100 were determined for a,b) HEK293 cells or c,d) MSCs by MTT assay.