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Bioreducible Hyperbranched Poly(amido amine)s for Gene Delivery

Jun Chen[#], **Chao Wu**, and **David Oupický**[*]
Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI 48202, USA

Abstract

A series of reducible hyperbranched poly(amido amine)s (RHB) with high transfection efficiency were designed and synthesized as non-viral gene delivery vectors. The polycations were synthesized by Michael addition copolymerization of *N*,*N*-dimethylaminodipropylenetriamine (DMDPTA) and two bisacrylamide monomers *N*,*N*′-hexamethylene bisacrylamide (HMBA) and *N*,*N*′-cystamine bisacrylamide (CBA). The density of disulfide linkages in the synthesized hyperbranched polymers was tuned by varying the feed molar ratio of the bisacrylamide monomers. The results demonstrate that disulfide content in RHB controls the molecular weight of the polycation degradation products, ease of polyplex disassembly, polycation cytotoxicity, and polyplex transfection activity. Due to their lower cytotoxicity, polyplexes based on reducible polycations could be used safely in a wider range of DNA doses than non-reducible controls. As a result, significantly increased transfection activity is achieved with optimized formulations of reducible polyplexes compared with non-reducible controls.

1. Introduction

Polyelectrolyte complexes of nucleic acids with polycations (polyplexes) represent promising non-viral alternatives for delivery of a variety of nucleic acid therapeutics.[1-3] While polyplexes offer numerous advantages, relatively low transfection activity and high cytotoxicity associated with the use of polycations continue to compromise their prospects. Recent developments in the synthesis of biodegradable polycations offer a viable solution for reduction of cytotoxicity and for improving transfection activity by controlling intracellular disassembly of polyplexes.[4-7]

In addition to hydrolytically degradable polycations, reducible polycations have been reported to significantly improve efficacy of the gene delivery process.[8] A redox potential gradient exists between extracellular environment and various subcellular organelles. Disulfide bonds present in the structure of polyplexes are easily reduced in the reducing intracellular environment, while preserved in the predominantly oxidizing extracellular space.[9-13] The intracellular cleavage of disulfide bonds in polyplexes is mostly mediated by thiol/disulfide exchange reactions with small redox molecules like glutathione (GSH); either alone or with the help of redox enzymes.[14] A recent report also suggests the possibility of a significant involvement of intracellular protein thiols, which represent even larger redox pool than GSH.[15] Regardless of the active agent, the reduction leads to enhanced rates of disassembly of the polycation-nucleic acid complexes, which is believed to increase intracellular bioavailability of the delivered nucleic acids [16-18] and to decrease polycation cytotoxicity.[12, 19-21]

* To whom correspondence should be addressed (oupicky@wayne.edu).
[#]Present address: Department of Textile Sciences, University of Manitoba, Canada

Hyperbranched polymers are highly branched macromolecules with three-dimensional dentritic architecture, low viscosity and a large number of functional groups, which provide for many potential applications in drug and gene delivery.[22-27] Although many linear reducible polycations have been described as gene delivery vectors,[28-32] there is only a limited number of reports on the use of well-defined hyperbranched reducible polycations.[5, 33]

Considering that degree of branching is known to affect transfection activity of polyplexes[27] and that branched polycations offer the additional benefit of higher molecular weights than similar linear polycations, further investigation of this class of gene delivery vectors is warranted. In this paper, we report gene delivery properties of a series of reducible hyperbranched poly(amido amine)s (RHB) with different disulfide content synthesized by Michael addition copolymerization.

2. Experimental section

2.1. Materials

N,*N*′-cystaminebisacrylamide (CBA) and *N*,*N*′-hexamethylenebisacrylamide (HMBA) were synthesized according to previously published protocols.[34] Dithiothreitol (DTT, Sigma), *N*,*N*-dimethyldipropylenetriamine (DMDPTA, Aldrich), branched polyethylenimine (bPEI, MW 25 kDa, Aldrich) were purchased in the highest available purity and used as received. Plasmid DNA, gWiz™ high-expression luciferase (gWiz-Luc), containing luciferase reporter gene was from Aldevron (Fargo, ND). All other reagents were from Fisher Scientific and used as received unless otherwise noted.

2.2. Polymer synthesis

Hyperbranched poly(amido amine)s were synthesized by Michael addition copolymerization of equal molar ratio of a triamine (DMDPTA) and bisacrylamide monomers (CBA and HMBA). Typically, CBA (0.260 g, 1.0 mmol) and HMBA (0.154 g, 1.0 mmol) were added into a small vial containing DMDPTA (0.318 g, 2.0 mmol) in methanol/water mixture (3.5 mL, 8/2 v/v). Polymerization was carried out for 3 days at 50 °C. Then, additional 0.1 mmol of DMDPTA was added to the reaction mixture to consume any residual acrylamide groups, and stirring was continued for 12 h at 50 °C. The polymers were isolated by freeze drying after extensive dialysis against distilled water acidified with HCl to pH 3 and a final dialysis against distilled water. Typical yield after dialysis was 30–40%.

2.3. Polymer characterization

^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian spectrometer (400 MHz). The number average (M_n) and weight-average (M_w) molecular weight and polydispersity index (PDI=M_w/M_n) were determined by size exclusion chromatography (SEC) system consisting of Shimadzu LC-10ADVP solvent delivery unit, CTO-10ASVP Shimadzu column oven and Polymer Labs PL gel 5 μm mixed C column. The system was also equipped with miniDAWN multi-angle light scattering detector and OPTILAB DSP interferometric refractometer (both Wyatt Technology, Santa Barbara, CA). Sodium acetate (300 mM, pH 4.5) was used as eluent at a flow rate of 1.0 mL/min and temperature of 30 °C. SEC data were analyzed using Astra 5.3.1.4 software from Wyatt Technology. Refractive index increments (dn/dc) of RHBs were determined by an interferometric refractometer and used in the SEC analysis.

2.4. Buffering capacity

The buffering capacity of the synthesized polycations was determined by acid-base titration. Briefly, 10 mL polymer solution with 10 mM total amine concentration was adjusted to pH 4.0 with 1 M HCl and the solution was titrated to pH 9.0 with 20 μL aliquots of 0.1 M NaOH

and pH was measured after each addition. Buffering capacity in the pH range of 7.4 to 5.1 was calculated as Δ[OH]/ΔpH, where Δ[OH] is the concentration difference after an aliquot of NaOH, and ΔpH is the corresponding increase in pH.

2.5. Agarose gel retardation

DNA polyplexes with polymer/DNA mass ratio of 5/1 were prepared by adding RHB in sodium acetate buffer solution (30 mM, pH 5.0) to a sodium acetate buffer solution (30 mM, pH 5.0) of plasmid DNA (20 μg/mL) followed by vortexing for 5 s and incubating at room temperature for 30 min. Samples were loaded onto a 0.8% agarose gel containing 0.5 μg/mL EtBr and run for 60 min at 120 V in 0.5X TBE running buffer. The gel was visualized under UV illumination on a Kodak Gel Logic 100 Imaging System.

2.6. Light scattering

Hydrodynamic diameter (D_H) and ζ potential of the polyplexes were determined by dynamic light scattering using Brookhaven ZetaPlus particle size and zeta potential analyzer. Static light scattering (SLS) measurements were performed with a DAWN EOS light scattering instrument (Wyatt Technology, USA) equipped with a 30 mW linearly polarized GaAs laser. The SLS analysis was conducted at an angular range θ = 22.5°–147° in 20 mL glass scintillation vials at 25 °C. The static light scattering data were analyzed by the second-order Debye fit (R(θ)/Kc vs.sin2(θ/2)) to obtain z-average of the root mean square radius (radius of gyration, R_G) of the polyplexes.

2.7. Transfection activity *in vitro*

Transfection experiments were performed with B16F10 mouse melanoma cells using gWiz-Luc plasmid. All transfection studies were performed in 48-well plates with cells plated 24 h before transfection at a seeding density of 40 000 cells per well. The polyplexes were prepared at w/w ratios of 3, 5, 10, 15, 20 and 40. DNA dose in each well in the transfection experiments was set in the range of 0.05, 0.1, 0.25, 0.5 and 1 μg/well. Only non-toxic formulations were tested (i.e., total RHB concentration lower than corresponding IC10). On the day of transfection, the cells were incubated with the polyplexes (25 μL in 150 μL of DMEM without FBS). After 3 h of incubation, the transfection mixture was removed and the cells were cultured for additional 24 h in fresh full DMEM media. To determine levels of luciferase expression, the culture medium was discarded and cell lysates harvested after incubation of cells for 30 min at room temperature in 100 μL of cell lysis reagent buffer (Promega). To measure the luciferase content, 100 μL of luciferase assay buffer (20 mM glycylglycine (pH 8), 1 mM $MgCl_2$, 0.1 mM EDTA, 3.5 mM DTT, 0.5 mM ATP, 0.27 mM coenzyme A) was automatically injected into 20 μL of cell lysate and the luminescence was integrated over 10 s using single tube Sirius luminometer (Zylux Corporation). Total cellular protein in the cell lysate was determined by the Bicinchoninic acid (BCA) protein assay using calibration curve constructed with standard bovine serum albumin solutions (Pierce). The luciferase transfection results are expressed as Relative Light Units (RLU) per mg of cellular protein. Unless stated otherwise, the results are expressed as mean RLU/mg of protein ± S.D. of triplicate experiments.

2.8. Cell viability

Cytotoxicity of RHB and control bPEI in B16F10 cells was determined by MTS assays using a commercially available CellTiter 96 aqueous cell proliferation assay (Promega). Thirty-thousand cells seeded in a 96-well plate 1 days before the experiment were incubated with increasing concentration of the polycations in 150 μL of DMEM/FBS. The medium was removed after 20 h and replaced with a mixture of 100 μL of fresh DMEM and 20 μL of MTS reagent solution. The absorbance of each sample was then measured at 505 nm to determine

cell viability. The results are expressed as the mean percentage cell viability relative to untreated cells.

3. RESULTS AND DISCUSSION

The main goal of this study was to prepare a novel series of reducible hyperbranched polycations designed to improve transfection efficacy by controlling polymer topology and content of disulfide bonds. Reducible polyplexes exploit intracellular reducing environment to improve the efficacy of the delivery of nucleic acids by selectively releasing the DNA in the subcellular space. GSH is the most abundant intracellular thiol present in mM concentrations inside the cell but only in μM concentrations in the blood plasma.[35] The majority of GSH is usually found in the cytoplasm (1–11 mM), which is also the principal site of GSH biosynthesis.[36-38] The most reducing environment in the cell is usually found within the nucleus, where it is required for DNA synthesis and repair and to maintain a number of transcription factors in reduced state.[39-41] The nuclear GSH levels are typically greater than those found in the cytoplasm and can reach up to 20 mM.[41-43] Another major pool of GSH in the cell is found in mitochondria (~5 mM).[44, 45] Both mitochondrial and nuclear GSH pools are at least partially independent of the cytoplasmic pool. Although easy intracellular DNA release from reducible polyplexes is believed to be a prerequisite for efficient transfection, some evidence suggests that too high disulfide content can actually decrease transfection activity due to premature disassembly of the polyplexes in the reducing intracellular environment, which leads to enhanced susceptibility to enzymatic degradation of the DNA.

Synthesis and characterization of reducible hyperbranched poly(amido amine)s

We employed Michael addition copolymerization of triamine DMDPTA monomer and two bisacrylamide monomers CBA and HMBA to prepare the RHBs with different content of disulfides by varying the bisacrylamide monomer molar ratio (Figure 1). In this type of Michael addition polymerization, the amine reactivity typically fits the following order: secondary amine (original) > primary amine >> secondary amine (formed). As a result, reaction of equal molar ratio of a triamine monomer and bisacrylamide monomers produces linear polymers.[46] The topology of these polymers can be also controlled by polymerization temperature as shown by Hong et al. who reported that the relative reactivity of the formed secondary amines is significantly enhanced by elevating reaction temperature above 48 °C, leading to the formation of hyperbranched polymers.[47] The rationale for selecting DMDPTA was to prepare polymers with tertiary amines in the side chain in order to improve binding with DNA. The resulting RHB then contain secondary amines and two types of tertiary amines: the backbone and side chain amines. Such a combination of different types of amines is often associated with high transfection activity.

The polymerization reaction was carried out in methanol/water solution at 50 °C for 3 days, when the reaction mixture became highly viscous. Before ending the polymerization, excess of DMDPTA was added to consume all the unreacted acrylamide double bonds in the final hyperbranched polymers. All polymers were dialyzed against distilled water acidified with HCl to remove low molecular weight fractions and to convert the polymers to their hydrochloride salts. From the ^{1}H NMR spectra, the molar ratios of CBA to HMBA in the final RHBs were close to molar ratios in the feed, indicating that the content of disulfide linkages in the final polymer can be easily controlled (Table 1). The branched structures of the synthesized polymers were confirmed by ^{1}H NMR and ^{13}C NMR (Figure 2). The disappearance of peaks between 5 and 6.5 ppm indicated quantitative conversion of the acrylamide groups in the final polymer products. The appearance of peaks at 49.5 ppm indicated the formation of secondary amines. However, the degree of branching could not be quantitatively determined from ^{1}H NMR and ^{13}C NMR due to overlap between the linear and

branched units signals. The molecular weight of the polymers was measured by SEC with light scattering detection (Table 1). The range of the number-average molecular weights (M_n) was from 12 to 20 kDa. The polydispersity index (PDI) was in the range from 1.4 to 2.7, indicating a good control of the polymerization and the lack of gel formation.

To confirm reduction of RHBs, the polymers were dissolved in PBS (pH 7.4) containing 20 mM DTT and changes of apparent molecular weight were followed by static light scattering (Figure 3). The molecular weight of all disulfide-containing RHBs decreased rapidly within the first minutes of degradation and did not change any further beyond 5 min. No molecular weight changes were observed in case of non-reducible RHB-0. The final molecular weight corresponds to the length of polymer chain between neighboring disulfide bonds and is controlled by disulfide content, degree of branching and polymerization conversion (molecular weight of the starting RHB). The final molecular weight will determine the rate of intracellular polyplex disassembly after complete reduction of RHB. Figure 3 confirms that increasing the disulfide content in RHB decreases the molecular weight of the degradation product. For example, RHB-100 degraded into fragments with M_w = 1570, while RHB-75 reached M_w = 3500 and RHB-50 M_w = 7900, respectively.

Buffering capacity is often believed to be an important factor for efficient intracellular delivery of polyplexes due to the proton sponge effect. Although the correlation between buffering capability and transfection activity has been questioned,[48] buffering capacity of polycations nevertheless remains an often studied property. Here, we determine buffering capacity as part of a broader evaluation of the effects of disulfides on the physicochemical properties of RHB. Buffering capacities were measured by acid-base titration in 150 mM NaCl aqueous solution (Figure 4). The results show that all RHBs have good buffering capability in the pH range of 5–7.4, which mimic the pH range from the high pH extracellular environment to the low pH endosomal environment. Increasing the disulfide content in RHB resulted in a small, but significant, decrease in buffering capacity.

Characterization of RHB/DNA polyplexes

The ability of the RHB polycations to condense DNA was confirmed by ethidium bromide exclusion and gel retardation assays (Figure 5). All RHBs were able to fully condense DNA at similar N:P ratios, suggesting that DNA binding properties are not influenced by the presence of disulfide bonds in the polycations (Figure 5a). Size and zeta potential of the polyplexes were measured by light scattering (Table 2). Size and surface charge of polyplexes depend strongly on polyplex composition only around the point of equivalency. When excess of polycation is used, the dependence on composition is weak. Therefore, we have selected mass ratio 10 as a representative composition to characterize the effect of increasing disulfide content in the polycations on the properties of their DNA polyplexes. As seen in Table 2, all RHBs formed polyplexes highly positively charged particles with similar sizes at w/w 10.

The sensitivity of RHB/DNA polyplexes to reducing environment was tested by following polyplex dissociation after reduction with DTT (Figure 5b). As the disulfide bonds in the RHB chains are cleaved in the reducing environment of DTT, the resultant short cationic residues then display lower affinity to the DNA and allow subsequent dissociation of the polyplexes by NaCl. The concentration of NaCl required to dissociate the polyplexes is directly related to the affinity between DNA and polycations, which in turn depends, among other factors, on the molecular weight of the polycations.[49, 50] Due to cooperative nature of polycation binding to DNA, the molecular weight dependence of the dissociating NaCl concentration is especially strong in the low-to-medium molecular weight range of the polycations. No detectable dissociation was observed under non-reducing conditions even in the presence of 300 mM NaCl. DNA should eventually be released from the polyplexes in the intracellular environment. When the polyplexes were incubated with 20 mM DTT, mimicking the intracellular

environment, DNA was fully released from RHB-100 and partly also from RHB-75 polyplexes. None of the remaining polyplexes could release DNA under reducing conditions and DNA migration in the gel remained fully retarded. This observation is explained by the differences in the molecular weight of the degradation products of RHBs as shown in Figure 3. Complete reduction of RHB-100 results in the shortest fragments, which are not capable of maintaining DNA in the condensed state even in the absence of NaCl. On the other hand, complete reduction of RHB-75 results in fragments with molecular weight about 3500. These fragments carry sufficient number of positive charges to interact with DNA and retard its gel migration in the absence of NaCl but the interactions are relatively weak and can be easily disrupted with the addition of 300 mM NaCl (Figure 5). Reduction of RHB-50 and -25 results in the formation of fragments that form polyplexes that require NaCl concentrations higher than 300 mM. DNA could be completely released from all polyplexes by incubation with heparin (not shown).

Transfection activity and cell viability

We first investigated the effect of chloroquine on transfection activity of RHB/DNA polyplexes (Figure 6). Chloroquine enhances transfection activity of polyplexes by several mechanisms, including: (i) pH buffering of endocytic vesicles (ii) protection of DNA against enzymatic degradation and (iii) displacement of polycations from DNA in the polyplexes.[51-54] The initial transfections in B16F10 mouse melanoma cells were conducted with RHB/DNA polyplexes prepared at w/w ratio 10 using the gWIZ-Luc plasmid (0.1 μg/well). The transfection activities of all the RHB/DNA polyplexes were 3–30-times higher than transfection efficiency of control bPEI polyplexes. Other reducible polycations have been reported to have comparable transfection efficiency to bPEI polyplexes. This result confirms that the series of RHB polymers represents a promising and highly effective gene delivery vectors.

The effect of chloroquine was determined in order to find out if the presence of disulfide bonds in RHBs affects intracellular trafficking of their DNA polyplexes. As shown in Figure 6, treatment with chloroquine increased transfection activity of all RHB polyplexes but RHB polyplexes with higher disulfide content showed higher sensitivity to chloroquine. For example, transfection activity of RHB-100, -75, and -50 polyplexes increased 3-6-fold, while activity of RHB-0 polyplexes showed no significant increase. It is also worth noting that no statistically significant differences in transfection activity among all the RHB polyplexes were observed in the presence of chloroquine. In comparison to other types of polycations, the sensitivity of the RHB polyplexes to chloroquine is relatively weak, suggesting effective intracellular delivery. Two factors most likely explain higher chloroquine sensitivity of RHB polyplexes with high disulfide content. First, the sensitivity to chloroquine could be an indication of less effective endosomal release. Considering that RHBs with higher disulfide content exhibit lower buffering capability and that the same polymers are more sensitive to chloroquine, this may suggest the differences stem from less effective proton sponge effect.

Second, the higher sensitivity could result from chloroquine enhancement of DNA stability against nuclease degradation. As shown in Figure 5, RHBs with high disulfide content disassemble and release free DNA easily in reducing environment. If the release occurs prematurely in the cytoplasm, the released DNA is susceptible to nuclease attack. Presence of chloroquine can stabilize the released DNA against nuclease degradation, thus increasing the transfection activity of those polyplexes that are more sensitive to nuclease degradation. Most likely, the combination of the two factors, less efficient endosomal release and less efficient protection against degradation, explains the higher chloroquine sensitivity of RHBs with high disulfide content.

Cytotoxicity of RHBs and control bPEI was evaluated by MTS assays in B16F10 cells (Figure 7). As expected, all the polymers containing disulfide bonds showed lower cytotoxicity than the nonreducible RHB-0. The IC50 value decreased from 50.6 ± 3.7 μg/mL for RHB-100 to

3.5 ± 0.2 μg/mL for RHB-0, which was comparable to the IC50 of the positive control bPEI (3.9 ± 0.2 μg/mL). Factors known to be involved in the cytotoxicity of cationic polymers include molecular weight, architecture, and charge density.[20, 55-58] In general, flexible polycations with high molecular weight and charge density exhibit high cytotoxicity. The RHB polymers used in this study have similar architecture and charge density, differing only in the content of biodegradable disulfide bonds and therefore in the molecular weight of their degradation products. The observed relationship between disulfide content and polycation cytotoxicity then suggests that degradation of reducible polycations in the intracellular reducing environment is responsible for the observed decrease in cytotoxicity. In addition to intracellular action, polycations exert their cytotoxicity by perturbing plasma membrane. It is expected that during the early stages of incubation, the plasma membrane disruption by extracellular polycations is a significant contributing factor for the observed cytotoxicity. Unlike approaches like PEGylation or decreasing the charge density, the presence of disulfide bonds in RHBs is unlikely to reduce the extracellular mechanism of cytotoxicity.

Based on the cytotoxicity results, we conducted detail evaluation of the transfection activity of the RHB polyplexes (Figure 8). The polyplexes were prepared at w/w ratios ranging from 3 to 40. DNA doses in the transfection experiments ranged from 0.05 μg/well to 1 μg/well. Only formulations considered safe were tested. For the purpose of this study, safe formulations were defined as those in which the total RHB concentration was lower than the corresponding IC10. The results in Figure 8 demonstrate clear benefits of reducible RHBs in the wider available range of formulations. Within the studied range of the two experimental parameters, the levels of luciferase expression vary within 5 orders of magnitude. Optimized formulations of RHB-100, -75, and -50 polyplexes demonstrate more than a 10-fold higher transfection activity than the best performing non-reducible controls (RHB-0). Lower cytotoxicity of RHB-100 and -75 allowed the use of their polyplexes in the entire DNA dose range. All RHBs exhibit strong dependence of transfection on DNA dose. For example, increasing the DNA dose 20-times (from 0.05 to 1 μg/well) results in 40,000-fold increase of luciferase expression in case of RHB-100 polyplexes. The effect of w/w ratio on transfection activity is different for RHBs with high disulfide content than for those with low disulfide content. In general, increasing the w/w ratio has relatively small effect on transfection activity of RHB-100 and RHB-75. On the other hand, polyplexes with lower or no disulfide content exhibit a strong dependence of w/w ratio and their transfection activity increases with increasing w/w ratio.

4. CONCLUSION

In summary, this work reports synthesis of a series of novel reducible hyperbranched polycations prepared by Michael addition polymerization. The density of reducible disulfide bonds can be easily tuned by varying bisacrylamide monomer molar ratios. The polymers exhibit good buffering capacity and nucleic acid binding ability. Cytotoxicity of the polycations is directly related to their reducible nature and decreases with increasing disulfide content. Due to their lowered cytotoxicity, the reducible polycations can be used safely in a wider range of DNA doses than non-reducible controls. As a result, significantly increased transfection activity is achieved with optimized formulations of reducible polyplexes; almost 200-fold higher than that of control bPEI polyplexes. The reported polycations represent a promising class of gene delivery vectors.

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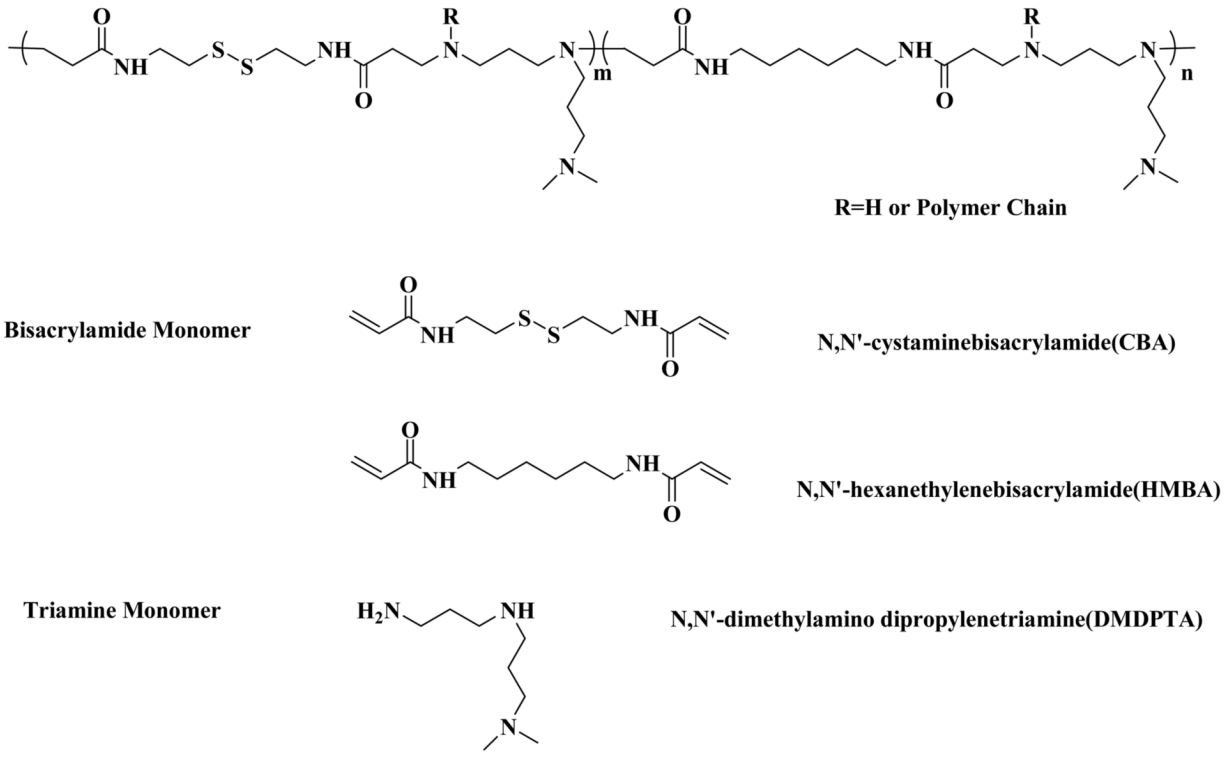

Figure 1.
Reducible hyperbranched poly(amido amine)s (RHB).

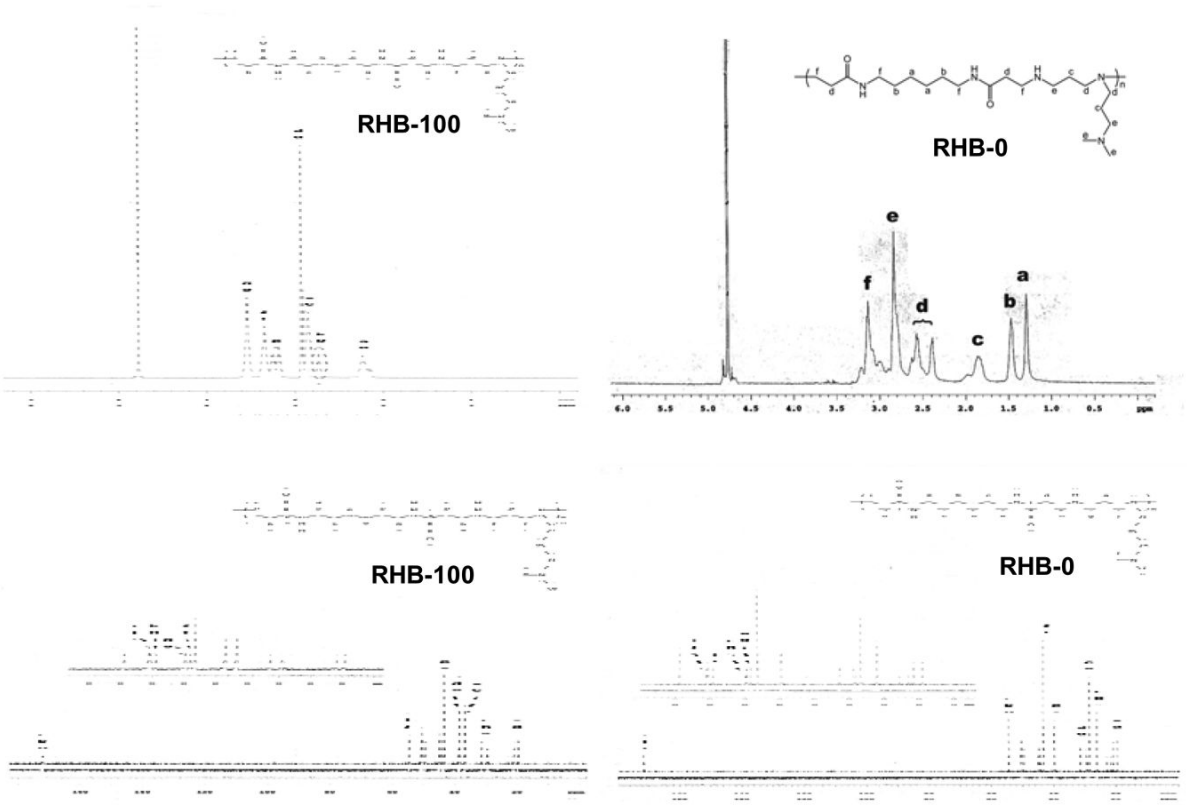

Figure 2.
^{1}H-NMR and ^{13}C-NMR spectra of RHB-100 and RHB-0.

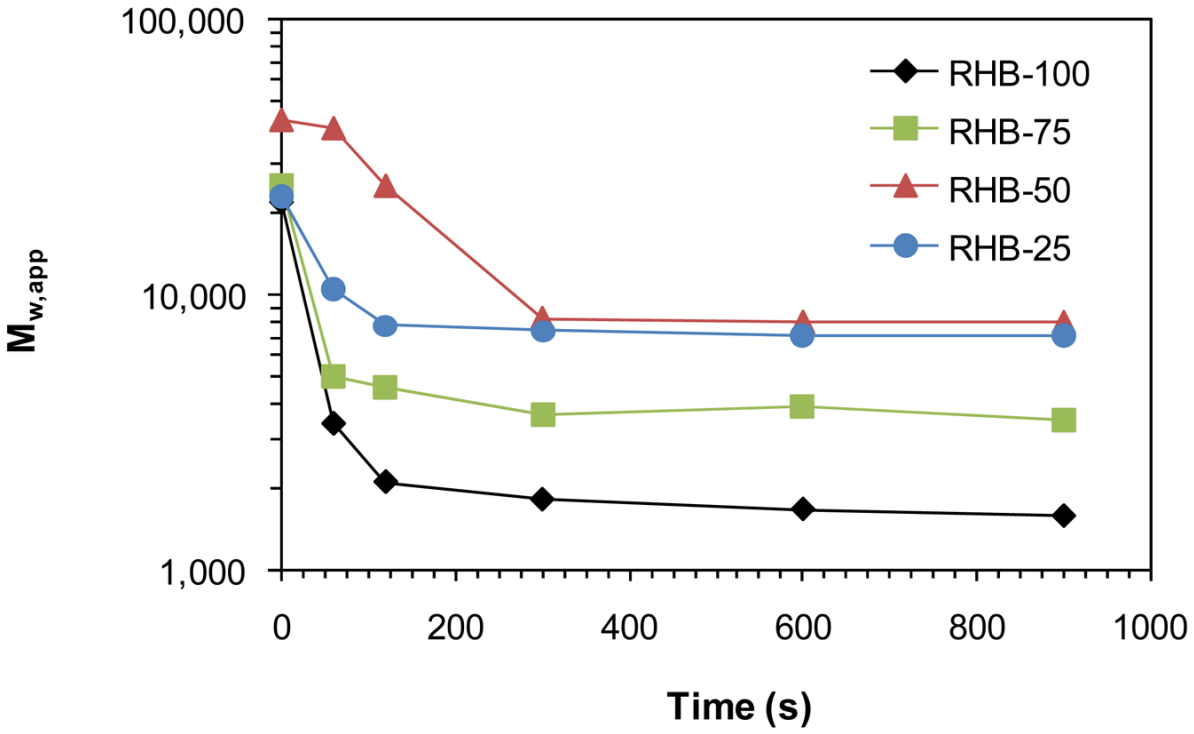

Figure 3.
Degradation of RHBs in 20 mM DTT.

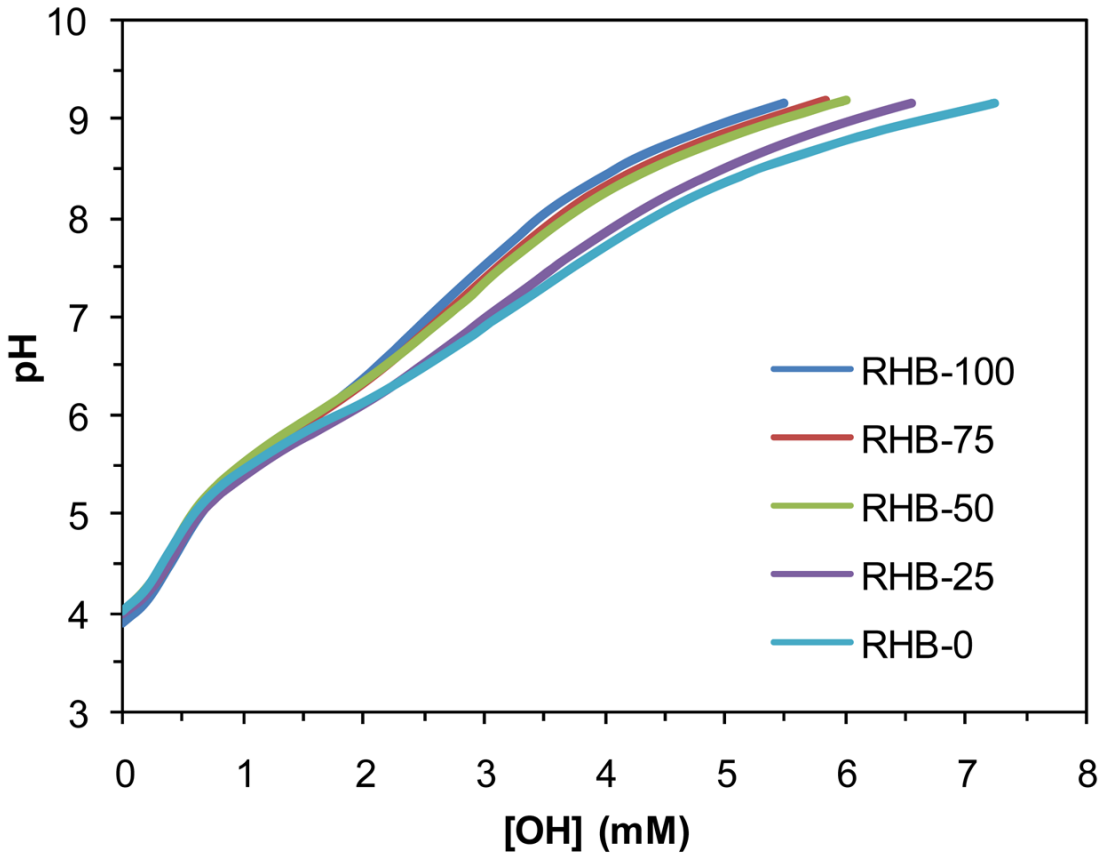

Figure 4.
Acid-base titration curves of RHBs.

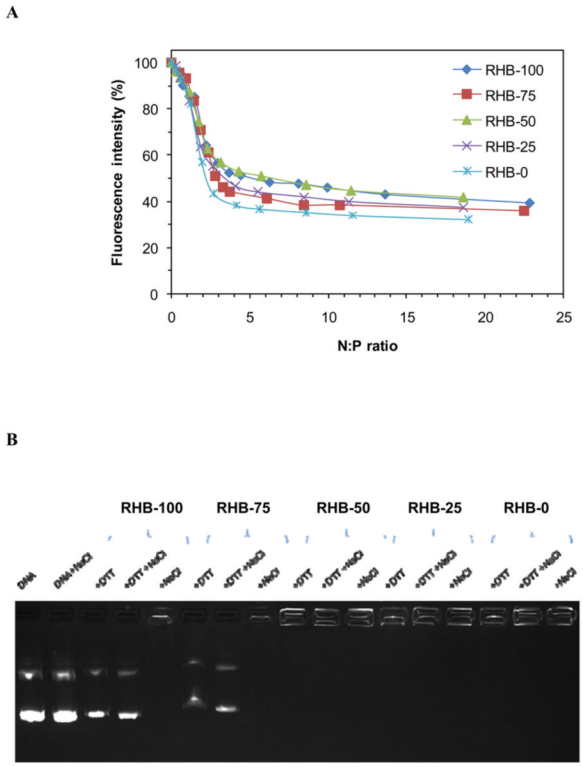

Figure 5.
DNA complexation by RHB. (A) Ethidium bromide exclusion assay. (B) Agarose gel electrophoresis of RHB/DNA polyplexes prepared at w/w 5/1 (± 20 mM DTT and ± 300 mM NaCl).

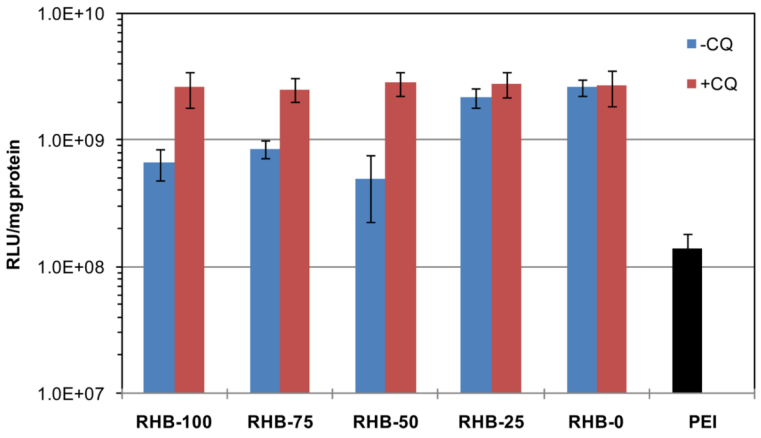

Figure 6.
Effect of chloroquine (CQ) on transfection activity of RHB/DNA polyplexes in B16F10 cells (0.1 μg DNA/well, ± 100 μM CQ, RHB/DNA w/w = 10, BPEI/DNA N/P = 10, RLU ±SD, n=3).

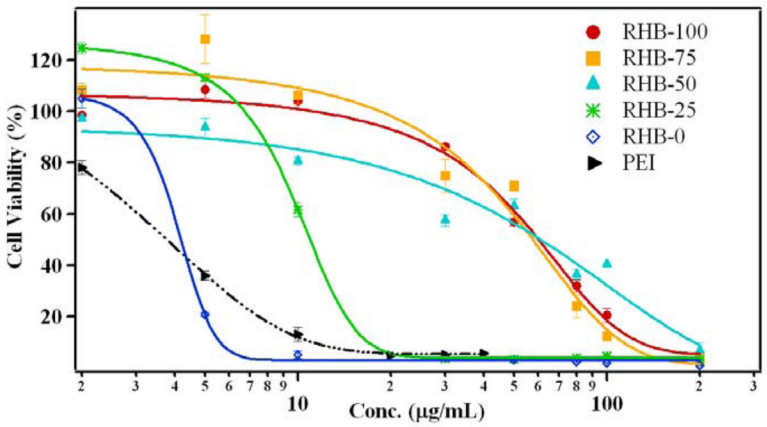

Figure 7.
Cell viability of RHBs in B16F10 cells.

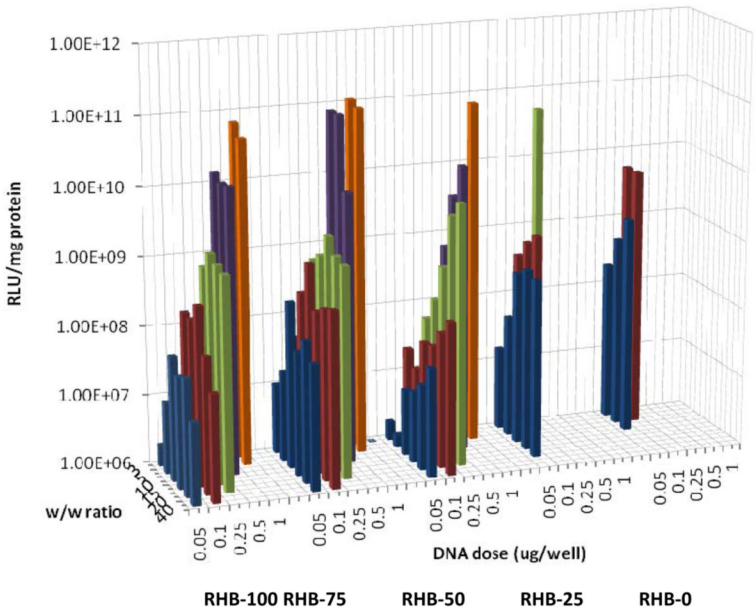

Figure 8.

Gene transfection matrix of RHB/DNA polyplexes in B16F10 cells. Polyplexes were formulated at w/w = 3–40 and transfection tested at DNA doses 0.05–1 μg/mL. Only non-toxic formulations were tested (i.e., total RHB concentration lower than corresponding IC10).

Table 1

Characterization of the synthesized polycations

Sample	CBA content (mol %)		M_w (kDa)	M_n (kDa)	PDI
	in feed	in polymer			
RHB-100	100	100	16.9	12.6	1.34
RHB-75	75	74	21.0	13.7	1.53
RHB-50	50	50	14.0	11.7	1.20
RHB-25	25	23	15.8	12.2	1.22
RHB-0	0	0	59.7	22.4	2.67

Table 2

Characterization of RHB/DNA polyplexes at w/w ratio 10/1

Polyplex	D_H (nm)	ζ potential (mV)	R_G (nm)
RHB-100/DNA	170.2	43.0	64.1
RHB-75/DNA	95.5	44.4	42.0
RHB-50/DNA	103.4	47.3	53.2
RHB-25/DNA	98.5	48.0	49.8
RHB-0/DNA	90.6	49.8	37.5

* SEM of all measurements was less than 5%