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Erythropoietin gene delivery using an arginine-grafted bioreducible polymer system

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Abstract

Erythropoietin (EPO) plays a key regulatory role in the formation of new red blood cells (RBCs). Erythropoietin may also have a role as a therapeutic agent to counteract ischemic injury in neural, cardiac and endothelial cells. One of the limitations preventing the therapeutic application of EPO is its short half-life. The goal of this study was to develop a gene delivery system for the prolonged and controlled release of EPO. The arginine grafted bioreducible polymer (ABP) and its PEGylated version, ABP-PEG10, were utilized to study the expression efficiency and therapeutic effectiveness of this erythropoietin gene delivery system *in vitro*. Poly(ethylene glycol) (PEG) modification of the ABP was employed to inhibit the particle aggregation resulting from the interactions between cationic polyplexes and the negatively charged proteins typically present in serum. Both the ABP and the ABPPEG10 carriers demonstrated efficient transfection and long-term production of EPO in a variety of cell types. The expressed EPO protein stimulated hematopoietic progenitor cells to form significant numbers of cell colonies *in vitro*. These data confirm that this EPO gene delivery system using a bioreducible polymeric carrier, either ABP or ABP-PEG 10, merits further testing as a potential therapeutic modality for a variety of clinically important disease states.

Keywords

Erythropoietin; Bioreducible Polymer; PEG; Gene Therapy; Hemolysis

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1. Introduction

Erythropoietin (EPO) plays a key regulatory role in the formation of new red blood cells (RBCs). Erythropoietin may also have a role as a therapeutic agent to counteract ischemic injury in neural, cardiac and endothelial cells [1]. Several reports have demonstrated the capacity of EPO to protect and revascularize the myocardium following ischemic injury [2-7]. One of the limitations preventing the therapeutic application of EPO is its short half-life. The development of a more sustained form of EPO with a longer half life would remove a significant barrier to its development as a therapeutic agent. Recently, several researchers have focused on the development of an EPO plasmid DNA delivered using either viral or non-viral carriers to promote the prolonged and controlled release of EPO *in vivo* [8-11].

Compared to viral vectors, polymer carriers offer several advantages for gene delivery *in vivo* including stability, non-immunogenicity, high loading capacity of plasmid DNA, and relative ease of large-scale production [12-14]. Cationic polymers such as poly(ethylenimine) (PEI), poly(L-lysine) (PLL), and poly(amidoamine)s dendrimers, however, are limited by toxicity related to their poor biocompatibility and biodegradability. To overcome these limitations, biocompatible and biodegradable polymers have been developed which are significantly less toxic [15, 16]. We have designed and synthesized several types of these biodegradable polymers [17-19]. In particular, we have developed an arginine-grafted bio-reducible polymer (ABP) with enhanced transfection efficiency and low cytotoxicity, due to the localizing ability of the arginine residues and the biodegradability of a reducible disulfide bond. We have previously reported on the use of this ABP for the delivery of siRNAs and plasmid DNA *in vitro* [17, 28].

Another important limitation of cationic polyplexes is their positive surface charge. This positive surface charge may interact with the negatively charged proteins present in serum [20, 21], resulting in particle aggregation and a reduction in transfection efficiency *in vivo*. To overcome this limitation, conjugation of poly(ethylene glycol) (PEG) to polycationic polymers has been employed. Several studies have demonstrated that PEGylation enhances carrier function in the presence of serum *in vivo*. To enhance the efficacy of our ABP carrier *in vivo*, we designed a PEGylated form of the ABP carrier, i.e. PEG_{5K}-ABP, and then studied the influence of various formulations of PEG_{5K}-ABP on polyplex formation, size, surface charge, serum stability, induction of hemolysis and transfection efficiency. We then tested the functionality of our PEG_{5K}-ABP carrier using the gene for erythropoietin in plasmid form (pEPO) to determine if this PEGylated ABP would result in enhanced gene delivery and the sustained release of erythropoietin. We carried out the transfection assays of the pEPO polyplex in a variety of cell types and analyzed the time-course release of EPO *in vitro*. A colony forming cell (CFC) assay was carried out to determine the biological activity of the expressed EPO protein. The cardio-protective effects of the released EPO were investigated by an apoptosis assay in rat cardiomyocyte H9C2 cells under hypoxic conditions using flow cytometry.

2. Materials and methods

2.1. Materials

N,N'-Cystaminebisacrylamide (CBA) was purchased from PolySciences, Inc. (Warrington, PA). Hyperbranched poly(ethylenimine) (bPEI25k), *tert*-Butyl-*N*-(6-aminohexyl) carbamate (*N*-Boc-1,6-diaminohexane, *N*-Boc-DAH), trifluoroacetic acid (TFA), triisobutylsilane (TIS), *N,N*-diisopropylethylamine (DIPEA), *N,N,N',N'*-tetramethylazodicarboxamide (TMAD) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), Dulbecco's modified Eagle's medium (DMEM), and

Annexin-V -fluorescein isothiocyanate (FITC) apoptotic kit were purchased from Invitrogen (Carlsbad, CA). Spectrapor dialysis membrane was purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA). Plasmid pCMV-Luc, containing a firefly luciferase reporter gene DNA (gWiz-Luc) was purchased from Aldevron, Inc. (Fargo, ND). Luciferase assay system and reporter lysis buffer were purchased from Promega (Madison, WI). Recombinant human EPO protein (rhEPO, Aropotin) was kindly provided by TS Corporation & Bioplant, Korea.

2.2. Synthesis and characterization of polymers

The arginine-modified bioreducible polymer, ABP, was synthesized by arginine modification into the primary amines of poly(CBA-DAH) as previously described. Briefly, synthesis of the poly(CBA-DAH) backbone was conducted via Michael reaction of equivalent moles of *N*-Boc-DAH and CBA in MeOH/H₂O solution (9:1, v/v), and the polymerization reaction was maintained under a dark nitrogen atmosphere at 60°C for 5 days. Then, 0.1 equivalent of *N*-Boc-DAH was added to terminate the polymerization by masking unreacted acrylamide groups and the reaction mixture was further stirred for 2 days at the same temperature. After the resulting product was precipitated with cold ether, Boc protecting groups of the product were removed by TFA 95% solution for 30 min in an ice bath. After de-protection, the reaction mixture was precipitated with diethyl ether, dialyzed using a dialysis membrane and then lyophilized. The synthesis of poly(CBA-DAH) was confirmed with proton NMR. To modify poly(CBA-DAH) in DMF with arginine residues, Fmoc-Arg(pbf)-OH (4 eq.), HBTU (4 eq.), and DIPEA (8 eq.) were added and the mixture was reacted overnight. The reaction was monitored with a Ninhydrin test. After the completion of the arginine modification, the crude mixture was precipitated to remove the unreacted and excess reagents with ethyl ether. The reactant was de-protected with 30% piperidine solution (DMF, V/V) for Fmoc and 95% TFA for pbf groups. After precipitation with cold ether, the crude product was dialyzed against water with the dialysis membrane (MWCO 1,000) followed by freeze drying. Arginine modification was confirmed with ¹H NMR, and an average molecular weight was determined by size exclusion chromatography (SEC). The average molecular weight was found to be approximately ~5 K.

For the PEG conjugation of ABP, 0.1 equivalent of Methoxy PEG 5K-NHS was added to the solution (pH 7.4, 0.1M PBS (0.15M NaCl, 2mM EDTA)) of ABP having about 10 residues of arginine. The reaction was carried out at room temperature for 2 hours. After reaction, the resulting crude product was precipitated to remove the unreacted and excess reagents with cold ethyl ether. The collected sample was purified using a dialysis membrane and then lyophilized. The conjugation of PEG was confirmed with proton NMR and size-exclusion chromatography (SEC, Superdex 75 column, calibrated with standard poly[N-(2-hydroxypropyl)-methacrylamide] (pHPMA)) using an AKTA FPLC system.

2.3. Preparation of plasmid hEPO DNA

The human erythropoietin (hEPO) cDNA was amplified by polymerase chain reaction using pDrive-hEPO (Open Biosystems, Huntsville, AL) as a template. The PCR primer sequences were as follows: forward primer, 5'-CCGGAATTCATGGGGGTGCACGAATGTC-3'; reverse primer, 5'-GCTCTAGATCATCTGTCCCCTGTCCTGCAG-3'. The EcoRI and XbaI sites were introduced to the PCR primers for cloning. The amplified hEPO cDNA was purified by agarose gel electrophoresis and elution. The hEPO cDNA was inserted into pCI (Promega, Madison, WI) at the EcoRI and XbaI sites, resulting in construction of pCMV-hEPO. The proper construction of the pCMV-hEPO was confirmed by direct sequencing. The constructed pCMV-hEPO (pEPO) was amplified in *E. coli* DH5α and purified using the Maxi plasmid purification kit (Qiagen, Valencia, CA). Purity and concentration of the

purified plasmid dissolved in TE buffer were measured using a Nanodrop 1000 spectrophotometer, and the purities at A260/A280 were 1.8 - 1.9.

2.4. Gel retardation assay

The pDNA condensation ability of ABP and ABP-PEG was assessed by an agarose gel electrophoresis assay. Agarose gel (0.8%, wt. /vol.) containing SYBR Safe DNA gel stain solution was prepared in TAE buffer. The solutions were prepared with pDNA (0.5 µg) and a corresponding amount of polymer in Hepes buffered saline, HBS (10 mM Hepes, 1 mM NaCl, pH 7.4). The two solutions were combined at various weight ratios (polymer/pDNA), slightly vortexed, and incubated for 30 min. After the loading dye was added, polyplex samples were loaded for electrophoresis and run for 15min at 120 V. In order to examine the DNase protection, samples of polyplexes were incubated with FBS (50%) for a designated period of time. After incubation, 1.5 µL of 10% sodium dodecyl sulfate (SDS) and 1.5 µL of loading dye were added into the aliquots of samples (10 µL). The treated samples were electrophoresed on an agarose gel (0.6%) containing SYBR Safe DNA gel stain. The bands of pDNA were detected by a UV Illuminator (Gel Documentation Systems, Bio-Rad, Hercules, CA).

2.5. Particle size and Zeta-potential measurements

The particle size and zeta-potential values of the polyplexes were measured using a Nano ZS (ZEN3600, Malvern Instruments) with a He-Ne ion laser (633 nm). 50 µL of polyplex solutions (0.5 µg of pDNA) were prepared at various weight ratios (polymer/pDNA) ranging from 1 to 40. After a 30 min incubation, polyplex solutions were diluted in filtered water to a final volume of 600 µL before measurement.

2.6. In vitro transfection experiments

For the transfection experiments, cells were plated at a density of 5×10^4 cells/well in 24-well plates in 500 µL media containing 10% FBS. After the cells were grown to 70-80% confluence, polyplexes were prepared using 0.5 µg pDNA at different weight ratios in HBS. After 30 min incubation, polyplexes (1µg pDNA in 1 mL) were added to the cells in the presence or absence of serum for 4h at 37°C. The media was then replaced with fresh DMEM containing 10% FBS. The treated cells remained in the 37°C incubator for 1 to 6 days. For the EPO protein assay, the culture medium containing hEPO protein was collected and centrifuged. The amount of EPO protein secreted from the transfected cells was measured using the Quantikine® IVD® human EPO immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For luciferase quantification, the cells were washed with PBS, treated with Reporter Lysis Buffer (Promega) and shaken for 30 min at room temperature. The expressed luciferase level was determined using a Luciferase assay system (Promega) on a luminometer from Dynex Technologies, Inc. (Chantilly, VA) and the amount of protein was quantified using a BCA Protein Assay Kit (Pierce, Rockford, IL).

2.7. Cytotoxicity

In vitro cytotoxicity was measured using an MTT assay. The experiments were performed in 24-well plates as described above for the transfection experiments. After incubation of the treated cells, 25 µL of stock solution of MTT (2 mg/ml in PBS) was added to each well. After 4 h incubation at 37°C, the media was carefully removed and 150 µL of DMSO was added. The absorbance was measured at 570 nm using a microplate reader (Model 680, Bio-Rad Lab, Hercules, CA). The cell viabilities were calculated as a percent absorbance to untreated control cells.

2.8. Hemolysis assay

The hemolytic activity of the ABP and the PEG-conjugated ABP was investigated using rat red blood cells. Fresh blood was collected in heparinized tubes from 12 week-old Sprague-Dawley rats, centrifuged at 1500 rpm for 10 min and washed three times with PBS to harvest the erythrocytes. The cell pellet was resuspended in PBS and the erythrocytes used within 24 h. 100 μ L of erythrocytes was mixed with 900 μ L of polymer solution dissolved in PBS at different concentrations. PBS and deionized water (DW) were used as controls. The mixtures were incubated with shaking at 37°C for 6 h. The cells were centrifuged at 1500 rpm for 5 min and then the collected supernatant was further centrifuged at 13000 rpm for 10 min. The released hemoglobin was measured at 410 nm using a Nanodrop 1000 spectrophotometer. The relative degree of hemolysis induced by the polymers was calculated by comparing the results to the degree of hemolysis induced by PBS (0%) and by DW (100%).

2.9. Colony forming assay

Freshly thawed peripheral blood mononuclear cells (PBMCs) supplied by SeraCare Life Sciences Inc. (Gaithersburg, MD) in 135 μ L of Cell Resuspension Media (IMDM containing 25% FBS) were seeded at a density of $\sim 1.0 \times 10^6$ cells/well in 6-well plates. Methylcellulose medium without EPO (StemCell Technologies, Vancouver, BC, Canada) was prepared with medium of pEPO /polymer (weight ratio 1:10) complex or control rhEPO. Expressed EPO proteins were adjusted to be 3 IU/ml at final concentration. 1.5 mL of prepared methylcellulose medium was added to each well and incubated at 37°C in a humidified incubator for 12 days. Colony forming capacity and morphology was measured using an EVOS microscope (AMG, Bothell, WA).

2.10. Annexin-V FITC apoptosis assay

For the apoptosis assay, a transfection experiment was conducted in a 6 well plate as described above for the transfection experiments. After transfection with the pEPO polyplex for 1 day, treated cells were placed under hypoxic conditions with 800 μ M hypoxia-mimic cobalt chloride (CoCl_2) for 20 h. Cells were then washed with PBS, detached with trypsin-EDTA and centrifuged. The harvested cells were resuspended and treated with FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Carlsbad, CA) for FACS analysis according to the manufacturer's protocol. After incubation of the cells at room temperature for 15 min in the dark, samples were analyzed with a BD FACScan analyzer (Becton Dickinson).

2.11. Statistical analysis

Statistical calculations were carried out using SPSS 12.0 software (SPSS Inc., Chicago, IL). Results were expressed as the mean value $\pm$ standard deviation. One-way analysis of variance (ANOVA) followed by Tukey/post hoc analysis was used to identify significance between groups.

3. Results and Discussion

3.1. Synthesis and characterization of polymers and construction of pCMV-hEPO

The arginine-modified bioreducible polymer (ABP) was used as the carrier for the EPO gene based on its previously reported high transfection efficiency and low cytotoxicity for the delivery of both siRNA and pDNA. ABP was synthesized as follows: first, poly(CBA-DAH) was obtained via Michael reaction of CBA and DAH. The primary amines of the poly(CBA-DAH) were then modified with arginine groups. The arginine modification was confirmed by ^1H NMR and found to be greater than 98%. To reduce the chance of aggregation of the

positively charged ABP with the negatively charged proteins present in serum, poly(ethylene glycol) (PEG) was conjugated to the bioreducible ABP. We designed and synthesized PEG_{5K}-ABP with a bioreducible disulfide bond. PEG conjugation was confirmed with proton NMR and found to be approximately 10% modification in the NMR spectrum (data not shown). The molecular weight of ABP-PEG_{5K} was also measured by SEC, and estimated to be about 10 K. To obtain the optimal PEG conjugation ratio for ABP, the effect of varying the relative PEG/ABP ratio on polyplex formation, size, surface charge, serum stability, induction of hemolysis and transfection efficiency was studied. The pCMV-hEPO was constructed using the CMV immediate-early promoter and enhancer and hEPO cDNA.

3.2. Characterization of polyplex formation and stability in serum

Prior to the investigation of the transfection efficiency of the pEPO/cationic polymer, the condensing ability of the ABP and ABP-PEG formulations with pDNA-EPO was examined by agarose gel electrophoresis. As shown in Fig. 1, pDNA migration with the ABP/pDNA polyplexes was retarded above a weight ratio of 2 while pDNA migration with the ABP-PEG 10 (ABP-PEG:ABP = 1:9) polyplex or the ABP-PEG 20 (ABP-PEG:ABP = 2:8) polyplex was retarded above a weight ratio of 5. These results demonstrate the capacity of both the ABP and the ABP-PEG formulations to sufficiently condense pDNA. The ability of PEGylation to protect pDNA against DNase (present in FBS) degradation was also determined using a gel electrophoresis assay. In order to assess and compare the protective effects of ABP and ABP-PEG, we performed the degradation experiments at a weight ratio of 5 for the ABP polyplex and 10 for the ABP-PEG/pDNA polyplex. After incubation of the polyplexes with FBS (50%), SDS was used to release the pDNA from the polyplexes to determine the integrity of the pDNA. As shown in the right panel of Fig.1, degraded pDNA was visualized by the presence of band 4 and by a decrease in the intensity of band 2 (intact pDNA). DNA and FBS were used as control samples and FBS/SDS was band 3. Plasmid DNA complexed with ABP began to degrade after 24 h of incubation with FBS. PEGylation, however, protected the pDNA up to 48 h at 37°C, with only slight pDNA degradation. These results show that PEGylation of the ABP can protect pDNA from nuclease degradation and improve the efficiency of gene delivery in the presence of serum. Fig 2 shows the average sizes and surface charges of the polyplexes measured by DLS. From a weight ratio of 5–40, the ABP polyplex particles showed positive Zeta-potential values converging at 30 mV. As expected, PEGylation of the ABP resulted in lower zeta-potential values than the ABP polyplex system alone. This reduced surface charge with PEGylation would be expected to reduce the interaction with the negatively charged proteins found in serum and improve transfection efficiency. After 6 h incubation in 50% FBS at 37°C, it was found that the PEGylated ABP polyplex was not as large as the ABP polyplex (Fig. 2). While the pDNA/ABP complex increased in size by 315%, the ABP-PEG 10 polyplex increased in size by only 144%. These results demonstrate that the conjugation of PEG to ABP can increase condensing ability with pDNA, protect against DNases and increase transfection efficiency in the presence of serum *in vivo*.

3.3. Hemolysis assay

In order to assess the biocompatibility of the ABP and ABP-PEG formulations as nanocarriers, a red blood cell (RBC) hemolysis assay was performed. Cationic polymers can interact with the negatively charged membranes of red blood cells [22, 23]. The release of hemoglobin as a result of hemolysis of red blood cells was used to assess the potential for disruption of the membranes of red blood cells by the ABP and ABP-PEG polymers. For controls, the hemolytic effect of de-ionized water (DW) and PBS was regarded as 100% and 0%, respectively. Erythrocytes were incubated with ABP and ABP-PEG 10 in the range of 0.02–1 mg/ml for 6 h at 37°C. As shown in Fig. 3, incubation with 200 µg/ml of ABP

resulted in hemolysis of 50% of the RBC, while incubation with the same concentration of 10% PEGylated ABP resulted in hemolysis of only 27% of the RBC. The high rate of hemolysis seen with the ABP is likely due to the higher positive charge properties of the ABP. Our results demonstrate that PEG conjugation to ABP reduces the rate of hemolysis of RBC and thereby improves the biocompatibility of ABP for *in vivo* applications.

3.4. In vitro transfection and cytotoxicity

3.4.1. Transfection and cytotoxicity of plasmid EPO using ABP—In order to investigate the suitability of ABP for EPO gene delivery, transfection efficiency assays were performed in various cell types, including HepG2 human hepatocellular carcinoma cells, HEK293 human embryonic kidney cells, H9C2 rat heart cells, A549 human lung epithelial cells and rat mesenchymal stem cells (rMSC). A branched PEI25k polyplex was used at a weight ratio of 1 as a control. Following transfection, supernatants from cells treated with each polyplex were analyzed to measure EPO expression by an ELISA assay. As shown in Fig. 4A, transfection with the pEPO/ABP complexes resulted in EPO protein expression greater than or equal to PEI25k in all cell types. We also carried out an MTT assay to determine the cytotoxicity of the pEPO/ABP polyplex on the HepG2, HEK293 and H9C2 cell types. PEI25k was again used as a control. Relative cell viability (RCV) was significantly decreased in three cell lines exposed to the PEI25k polyplex at a weight ratio of 1, indicating significant cytotoxicity. In contrast to PEI25k, the cell lines exposed to the pEPO/ABP polyplexes displayed high RCV (above 85%) up to a weight ratio 20 (Fig. 4B). Based on these results, we concluded that the plasmid EPO gene can be delivered with low cytotoxicity using the ABP. In order to determine the extent of sustained EPO transgene expression, the time dependent secretion of EPO was measured in the following cell types: H9C2, rMSC, HepG2 and HEK293. Three days after treatment, increased EPO levels were achieved and reached a plateau at approximately 1 week (Fig. 5). These results demonstrate that the plasmid EPO gene complexed with the ABP can achieve significant, long-term production of EPO without adverse effects *in vitro*. The initial high release of EPO followed by sustained gene expression of EPO may be applicable to a number of clinically important disease states.

3.4.2. The effect of PEGylation on transfection efficiency and cytotoxicity—In order to investigate the carrier function and potential advantage of the PEG formulation in the presence of serum, a transfection assay with pEPO and the pCMV-Luc reporter gene was performed in kidney cells: NRK (normal rat kidney) 52E cells and HEK293 cells which normally produce EPO. While there was no significant difference between ABP and ABPPEG 10 in the absence of serum, there were quite different gene transfection results in the presence of 10% serum (Fig 6A). ABP-PEG formulations were associated with a 3-fold reduction in luciferase gene expression and no noticeable decrease in EPO expression, while use of the ABP polyplex was associated with a 10-fold reduction in luciferase activity and an approximately 30% decrease in EPO production with the addition of serum. These results suggest that PEG conjugation may maintain stability of the polyplexes in serum, an important consideration for systemic delivery. To evaluate the transfection efficiency of PEGylation at various weight ratios, the NRK and 293 cells were used. Figure 6B shows that ABP-PEG efficiently enhanced gene expression of EPO (a) and Luciferase (b) in the presence of serum. Transfection with the ABP polyplexes resulted in considerably reduced expression when compared with ABP-PEG 10. The ABP-PEG 10 complexes demonstrated high transfection efficiency with significantly reduced toxicity (Fig 6C). ABP is a much less toxic polymeric carrier compared to a representative cationic polymeric carrier such as PEI [17]. ABP itself, however, was associated with 60% cell viability at high concentration (100 μ g/mL) and the ABP polyplex was associated with toxicity at a weight ratio of 40. The ABP-PEG polyplexes demonstrated a marked improvement in viability in the presence of

both cell types even at high weight ratios. Based on these results, ABP-PEG 10 may be the optimal formulation for transfection in the presence of serum.

3.5. Biological functional analysis of expressed EPO protein

3.5.1. Colony forming assay—In order to investigate the functionality of the released EPO protein after transfection with the ABP pCMV-EPO polyplexes, a colony forming assay (CFA) was performed with peripheral blood mononuclear cells (PBMCs) [24–27]. An *in vitro* colony forming assay is a direct and reliable measure of the ability of erythropoietin to stimulate proliferation and differentiation of hematopoietic progenitors to form colonies of differentiated cells in a semi-solid methylcellulose media. The colony forming assay was carried out in PBMCs with a human methylcellulose complete media without EPO. Recombinant human EPO protein (rhEPO) was used as a positive control. After 2 weeks incubation, formed clusters or colonies were observed using light microscopy. Figure 7 shows the cell colonies formed following exposure to rhEPO (positive control), non-exposure to rhEPO (negative control), the ABP pCMV-EPO polyplex and the ABP-PEG 10 pCMV-EPO polyplex. This data demonstrates that transfection with the ABP EPO polyplex and the ABP-PEG 10 EPO polyplex results in functional EPO protein expression that exceeds the positive control of rhEPO in stimulating production of colony forming units.

3.5.2. Anti-apoptotic activity in cardiomyocytes—Several studies have demonstrated that EPO can protect the heart and other organs from ischemic injury [2–7]. To assess the anti-apoptotic activity of the expressed EPO protein, an Annexin V-FITC apoptosis detection kit was used to determine the degree of apoptosis present under hypoxic conditions in rat H9C2 cells. The use of a hypoxic mimic agent, CoCl_2 (positive control), decreased the percentage of viable cells present, as identified by annexin V and PI negative staining (left and bottom panel), from 74.2% to 12.8% (Fig. 8). EPO gene transfection with the ABP polyplex or the ABP-PEG 10 polyplex significantly increased the percentage of viable cells present to near the positive control levels of rhEPO with Annexin-V staining. This anti-apoptotic effect suggests that EPO gene therapy may have a protective effect on the myocardium under ischemic conditions *in vivo*.

4. Conclusions

The arginine-modified bioreducible polymers, ABP and PEGylated ABP (ABP-PEG10), mediated efficient gene transfer with low cytotoxicity. ABP-PEG10 can be an effective polymer gene carrier *in vivo*, especially for systemic delivery by protecting against nuclease degradation. These polymers can effectively transfect pCMV-hEPO into a variety of cell types with long-term EPO expression. In addition, the EPO protein produced can stimulate hematopoietic progenitor cells and inhibit cardiomyocyte apoptosis *in vitro*. Gene delivery using a bioreducible polymer carrier, either ABP or ABP-PEG10, may be a promising approach to expand the therapeutic application of EPO to a greater range of clinically important disease states.

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Fig. 1. Gel retardation (A) and DNase protection of plasmid DNA with 50% serum (B). Band 1 is the location of the loading sample. Band 2: released and supercoiled pDNA; 3: combination of FBS and SDS; 4: degraded pDNA. As a control, buffer without DNA was incubated in the presence of FBS and SDS.

Fig. 2.

Average size and Zeta potential measurement of ABP and ABP-PEG. Arrows indicate the increase in the size of the ABP polyplex (red) and ABP-PEG 10 polyplex (black) in the presence of 50% serum.

Fig. 3. Hemolysis assay. The UV spectrum (A) and percentage (mean $\pm$ SD) of released hemoglobin from red blood cells (B) after 6 h incubation with different concentrations of ABP and ABP-PEG 10.

Fig. 4. Transfection efficiency (A) and MTT assay results (B) in a variety of cell types without serum. Data in 4(A) and along the x-axis of 4(B) represent the weight ratios of the ABP polyplexes. PEI25k was used as a control. Relative cell viability (RCV) is measured as a (%) to untreated cells.

Fig. 5.
The time course of EPO protein release in different cell types treated with pEPO/ABP complexes in the absence of serum. Cell supernatants were analyzed by an ELISA.

Fig. 6.

Transfection and toxicity results in NRK and 293 cells with ABP and ABP-PEG carriers in the presence of serum. (A) Gene transfection efficiency of EPO DNA (a) and luciferase gene (b) at a weight ratio of 20 in the presence or absence of serum. (B) The transfection results of EPO DNA (a) and luciferase gene (b) at varying weight ratios in the presence of 10% FBS. The data presented represent the weight ratios of the ABP polyplexes. (C) Relative cell viabilities of polymer (a) and polyplexes (b) in NRK and 293 cells.

Fig. 7. Colonies formed by EPO produced by gene transfection in PBMCs. rhEPO protein was used as a positive control.

Fig. 8.
Anti-apoptotic potential of EPO expressing rat cardiomyocytes in H9C2 cells.
Representative Annexin-V/PI flow cytometry and percentage of viable cells (Annexin V, PI negative) under hypoxic conditions.