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Cystamine-terminated poly(beta-amino ester)s for siRNA delivery to human mesenchymal stem cells and enhancement of osteogenic differentiation

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Abstract

Enhancing human mesenchymal stem cell (hMSC) differentiation via RNA interference (RNAi) could provide an effective way of controlling cell fate for tissue engineering, but a safe and effective delivery vehicle must first be developed. Here, we evaluated an array of synthetic end-modified poly(beta-amino ester) (PBAE)-based nanoparticles to optimize siRNA delivery into hMSCs. In general, cystamine-terminated polymers caused the most knockdown, with the best polymer achieving 91% knockdown 20 days post-transfection. Binding studies revealed that the cystamine-terminated polymer bound siRNA tightly at lower weight ratios of polymer to siRNA but then efficiently released siRNA upon exposure to a reducing environment, suggesting that this class of PBAEs can form tight initial interactions with its cargo and then cause efficient, environmentally-triggered release in the cytoplasm. Finally, we tested a functional application of this system by transfecting hMSCs with siRNA against an inhibitor of osteogenesis, B-cell lymphoma (Bcl)-like protein 2 (BCL2L2). This resulted in enhanced osteogenesis over 4 weeks as evidenced by Alizarin Red S staining and calcium quantification. The bioreducible PBAE/siRNA nanoparticles developed here can provide a means of safe and effective control of hMSC differentiation for a wide variety of applications.

Keywords

siRNA delivery; Nanoparticle; Mesenchymal stem cell; Genetic engineering; Bone tissue engineering

Introduction

RNA interference (RNAi) and other forms of gene delivery hold great potential in directing stem cell fate. Safe and effective materials, however, must first be identified for delivery to the relevant cell types. Bone marrow-derived mesenchymal stem cells (MSCs) can differentiate into multiple lineages, including cartilage and bone [1]; MSC-based therapies are, therefore, an active area of research for applications like cartilage repair [2] and bone regeneration [3]. The strategy of modulating MSC gene activity *ex vivo* prior to

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transplantation, could allow tissue engineers to control MSC fate better than in an *in vivo* system [4] while still bypassing long *in vitro* culture times [3] by increasing the efficiency of differentiation down a specified lineage. While viral transduction can yield high efficiencies in genetic engineering of MSCs [5], viral vectors bring with them several safety concerns and limitations in the size and type of cargo they carry [6]. Non-viral gene delivery, while potentially bypassing such concerns [7], is generally less effective [8]. Primary human cells, including MSCs, tend to be difficult to transfect with non-viral delivery methods [9]. Although electrical approaches, like nucleofection, can be employed [10], both nucleofection and traditional electroporation still often cause heavy cell death [11]. These considerations have prevented gene therapy from seeing widespread clinical use in areas such as bone regeneration [12].

Lipid-based materials can be effective in siRNA delivery. Such materials generally form liposome-like vehicles within which siRNA can be encapsulated and then carried [13]. Several groups have capitalized on this property of cationic lipid formulations for siRNA delivery [14, 15], using both natural and synthetic lipids to facilitate cellular entry via endocytosis or membrane fusion [16]. Because of the prevalence of their use in the field of siRNA delivery, although lipids are not a focus of this report, a lipidic product is used as a control in these studies. In particular, we compare our results here against results from Lipofectamine™ 2000, which has been widely used in the gene delivery literature as a leading commercial transfection agent. However, many lipid-based materials can be prohibitively cytotoxic [17] and unstable, especially in the presence of salts or serum [18]. The commonly used cationic polymer polyethyleneimine (PEI) must be chemically modified to avoid cytotoxicity [19] and, along with other methods like gold nanoparticle immobilization, also requires high siRNA doses of up to 200 nM [20, 21], even when using easier-to-transfect cell types such as CHO-K1 or HeLa cells.

Poly(β -amino ester)s (PBAEs) are an attractive non-viral method of gene delivery, as they are simple to synthesize, easily chemically modified, and hydrolytically degradable under physiological conditions [22–24]. We have previously shown success in DNA delivery to MSCs [25], but the ability to deliver other biomolecules such as siRNA would expand the flexibility of the cationic polymer delivery system by allowing gene knockdown as well as upregulation. Other researchers have previously been unsuccessful in transfecting cells with PBAE-siRNA nanoparticles without the use of gold nanoparticles as a substrate [26]. Researchers using similar systems have used high siRNA doses of up to 125 nM, usually in the absence of serum in the transfection media, to achieve moderate knockdown [27, 28]. Our early studies of siRNA delivery to another hard-to-transfect primary human cell type have shown that polymers ideal for DNA delivery may not be the same as those necessary for siRNA delivery [24], likely due to differences in chemical and physical properties of the two types of nucleic acids as well as differences in their mechanisms of action [29]. As such, PBAEs optimized specifically for siRNA delivery must be identified. Here, we synthesize, characterize, and evaluate an array of PBAEs for effective siRNA delivery to primary human MSCs (hMSCs). The ability to screen many PBAE formulations in a high-throughput manner provides a powerful tool to not only quickly identify top formulations but also see trends in the relationship between polymer structure and function, thereby helping to further refine results and to direct studies in similar systems. This bionanotechnology for siRNA delivery can be an enabling tool for control-based approaches to engineering biological systems [30].

As a model for knockdown, we transduced hMSCs with lentivirus to overexpress enhanced green fluorescence protein (eGFP). We then transfected these cells with siRNA against eGFP (siGFP) using PBAEs, and monitored eGFP expression over time as a measure of knockdown efficiency. We ensured the transduced hMSCs had not lost their potency by

evaluating multilineage differentiation to ensure their suitability for future biological applications. Finally, to demonstrate the potential applications of our approach, we identified the leading nanoparticle formulation among those we developed and tested, utilized this formulations to deliver siRNA against an inhibitor of osteogenesis, B-cell lymphoma (Bcl)-like protein 2 (BCL2L2), and examined the effect on hMSC osteogenic differentiation.

Materials and Methods

Materials

Monomers used for polymer synthesis (Fig. 1) include 1,3-propanediol diacrylate (B3; Monomer-Polymer and Dajac Labs, Trevose, PA); 1,4-butanediol diacrylate (B4; Alfa Aesar, Ward Hill, MA); 1,5-pentanediol diacrylate (B5, Monomer-Polymer and Dajac Labs); 1,6-hexanediol diacrylate (B6, Alfa Aesar); 3-amino-1-propanol (S3, Alfa Aesar); 4-amino-1-butanol (S4, Alfa Aesar); 5-amino-1-pentanol (S5, Alfa Aesar); 6-amino-1-hexanol (S6, Sigma Aldrich, St. Louis, MO); 1,3-diaminopropane (E1, Sigma Aldrich); 1,3-diaminopentane (E3; TCI America, Portland, OR); 2-methyl-1,5-diaminopentane (E4, TCI America); 2-(3-aminopropylamino)ethanol (E6, Sigma Aldrich); 1-(3-aminopropyl)-4-methylpiperazine (E7, Alfa Aesar); 1-(3-aminopropyl)pyrrolidine (E8, TCI America); and cystamine dihydrochloride (E10, Alfa Aesar). siRNA against eGFP (siGFP) with 5'-CAAGCUGACCCUGAAGUUCTT (sense) and 3'-GAACUUCAGGGUCAGCUUGCC (antisense) (Silencer® positive control) and a scrambled siRNA sequence (scrRNA) with 5'-AGUACUGCUUACGAUACGGTT (sense) and 3'-CCGUAUCGUAAGCAGUACUTT (anti-sense) (Silencer® negative control #1) were designed by and purchased from Ambion, Inc. (Carlsbad, CA). For viral transduction, lentivirus containing pPPT-eGFP plasmid was received from Dr. David Yue (Johns Hopkins University). Lipofectamine™ 2000 and Opti-MEM I were from Invitrogen (Carlsbad, CA) and used according to manufacturer instructions. For gel electrophoresis, UltraPure™ agarose was purchased from Invitrogen. All other materials used were reagent grade.

eGFP-lentiviral transduction of hMSCs

Lentiviruses containing pPPT-eGFP plasmids (CMV promoter) were diluted in serum-free medium (Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) with high glucose supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Cellgro, Manassas, VA)) and added to passage 1 hMSCs (Lonza (formerly Cambrex), Walkersville, MD) at 10% confluency. Complete expansion medium (the above mixture supplemented with 10% v/v fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA)) was added 4 hours after transduction to dilute viral titer, and medium was replaced with fresh complete medium after 48 hours. Fluorescence microscopy and flow cytometry were used to confirm the overexpression of eGFP; we obtained an efficiency of > 99% (Supplementary Fig. S1).

hMSC pre-differentiation and pre-transfection expansion

hMSCs were grown at 37 °C, 5% CO₂, and 21% O₂. All differentiation studies were done at passage 5. hMSCs were expanded in monolayer at passage 4 with high glucose DMEM, 10% v/v FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, and 1 ng/mL basic fibroblast growth factor 2 (bFGF-2; PeproTech, Rocky Hill, NJ). At 80% confluency, cells were released from monolayer via trypsin and placed under differentiation conditions.

hMSC adipogenic differentiation

Previously expanded hMSCs were plated at a density of 10000/cm² and cultured for 4 weeks with high glucose DMEM, 10% v/v FBS, 100 U/mL penicillin and 100 µg/mL streptomycin,

5 $\mu\text{g/mL}$ recombinant human insulin (Invitrogen), 1 μM dexamethasone (Sigma Aldrich), 200 μM indomethacin (Sigma Aldrich), and 500 μM 3-isobutyl-1-methylxanthine (IBMX; Sigma Aldrich)) [1]. Cultures were assayed for adipogenesis by staining lipid droplets with Oil Red O (Sigma Aldrich).

hMSC osteogenic differentiation

Previously expanded hMSCs were plated at a density of 5000/ cm^2 and cultured for 4 weeks with low glucose DMEM, 10% v/v FBS, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin, 10 mM β -glycerophosphate (Sigma Aldrich), 100 nM dexamethasone (Sigma Aldrich), and 50 μM ascorbic acid (Sigma Aldrich) [31]. Cultures were assayed for osteogenesis by staining protein-associated calcification with Alizarin Red S (Sigma Aldrich).

Polymer synthesis

Polymers were synthesized using previously reported protocols and are referred to following the reported nomenclature, with a polymer's name referring directly to its constituent monomers [23, 32, 33]. The number following the "B" title refers to the number of carbons between acrylate groups in the backbone monomer and the number following the "S" title refers to the number of carbons between the amine group and the alcohol group in the side chain. One backbone diacrylate monomer (B3, B4, B5, or B6) was mixed with one sidechain monomer (S3, S4, S5, or S6) at a 1.2:1 molar ratio of B:S. The reaction was carried out with stirring for 24 hours at 90 °C to allow formation of the base polymer (B-S). The resulting base polymer was dissolved in dimethyl sulfoxide (DMSO) at 167 mg/mL. 480 μL of B-S in DMSO was mixed with 320 μL of a 0.5 M solution of one endcapping monomer (Fig. 1: E1, E3, E4, E6, E7, E8, E10) and vortexed for 1 hour at room temperature. For E10-capped polymers, the resulting B-S-E polymer was incubated at room temperature for 1 day to ensure completion of the reaction before storage at -20 °C. All other polymers were immediately stored at -20 °C after endcapping. For the remainder of this report, polymers will be referred to by their composition (e.g. B4-S3-E10 or 4310).

All synthesized polymers used in this study were dissolved in BHT-stabilized tetrahydrofuran with 5% DMSO and 1% piperidine, filtered through a 0.2- μm filter, and measured with gel permeation chromatography (GPC; Waters, Milford, MA) to determine molecular weight. Representative polymers were diluted with deuterated DMSO and also analyzed by NMR (400 MHz, Bruker, Billerica, MA) using TopSpin 2 software (Bruker) for spectrum acquisition and ACD/NMR Processor (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) to verify the completion of both steps of the reaction.

Nanoparticle characterization: Nanoparticle Tracking Analysis (NTA)

siRNA was diluted to 10 $\mu\text{g/mL}$ in 25 mM sodium acetate buffer (pH 5). Polymer was diluted in the same sodium acetate buffer and added to the siRNA solution in a 1:1 volume ratio, for a final polymer-to-siRNA weight ratio (w/w) of 100–200 (500–200 $\mu\text{g/mL}$ final polymer concentration). This solution was mixed by pipetting and then incubated at room temperature for 10 minutes to allow self-assembly to occur. The resulting nanoparticle suspension was diluted 1:100 in phosphate-buffered saline (PBS), then measured using a NanoSight NS500 (NanoSight Ltd., United Kingdom) and analyzed with NanoSight NTA 2.2 software as previously described [34]. Reported values for size are number-weighted hydrodynamic diameters of the particles.

Polymer-siRNA binding assay: gel electrophoresis

Gel retardation assays were carried out by adding polymer of varying concentrations in sodium acetate buffer to a constant concentration of siRNA in sodium acetate, as with

normal particle preparation protocols (above). After 10 minutes of incubation, a solution of 30% v/v glycerol in water was added at a 1:5 v/v ratio as a loading buffer. Dyes were not added to the loading buffer, as they were found to interfere with binding. Samples were loaded into a 1% agarose gel with 1 $\mu\text{g/mL}$ ethidium bromide at 125 ng siRNA per well. Samples were run for 15 min under 100 V, then visualized using UV exposure. Lack of a visible band at the location corresponding to siRNA alone (0 w/w polymer) was considered complete complexation of the nucleic acid by polymer.

To test the effect of a reducing agent, after 10 minutes of complexation, PBS buffer alone or PBS with L-glutathione (GSH) was added to each tube of particles, with a final GSH concentration of 5 mM to match approximate intracellular concentration of 1–10 mM [35]. Samples were immediately loaded into a 1% agarose gel and run for 15 minutes under 100 V before visualization under UV.

Transfection of hMSCs with siRNA against eGFP

GFP⁺ hMSCs were seeded in clear-bottom, opaque-sided 96-well plates at a density of 10000/ cm^2 and allowed to adhere overnight. All experiments were carried out on passage 3–6 hMSCs. Before transfection, the GFP signal from each well was measured using a fluorescence multiplate reader (Synergy 2, Biotek, VT). Background signal was subtracted using GFP[−] hMSCs seeded alongside the GFP⁺ cells in complete expansion medium as described above. siRNA against eGFP (siGFP; Ambion, CA) was diluted in 25 mM sodium acetate buffer (pH 5).

Polymer was diluted in sodium acetate and added to the siRNA solution in a 1:1 volume ratio, for a final polymer-to-siRNA weight ratio (w/w) of 100–200 w/w. This solution was mixed by pipetting and then incubated at room temperature for 10 minutes to allow self-assembly to occur. The resulting nanoparticles were added to cells in complete expansion medium for a final siRNA dose of 60 nM (0.9 $\mu\text{g/mL}$) and final polymer dose of 90–180 $\mu\text{g/mL}$. Studies were done in triplicate. Cells were incubated with the particles for 2 hours before the media were changed and replaced with fresh, complete medium. For each polymer tested, one group was treated with siGFP and another with scrambled siRNA (scrRNA; Ambion, CA) complexed with the same polymer as a paired control. A replicate set of wells was prepared for each group for viability measurements.

After 24 hours, viability was measured using an MTS assay (CellTiter Aqueous One, Promega, Madison, WI) according to the manufacturer's instructions. On the remaining plates, medium was changed every 3–4 days and fluorescence measurements were taken daily. At each timepoint, knockdown was calculated by subtracting background signal, then calculating the ratio of signal in the siGFP group to that in the scrRNA group and subtracting this ratio from 1. For particles with the highest activity, cells were trypsinized after 12 days, resuspended in PBS with 2% FBS, and analyzed by flow cytometry (Accuri C6; Accuri Cytometers, MI) to confirm readings taken using the plate reader. The median of the FL1 signal (emission 530/30 nm) was calculated for each group of cells using FlowJo 7 (Treestar Inc., Ashland, OR). Background fluorescence, or the median of GFP[−] cells, was subtracted from all values, and the ratio of signals from scrRNA- and siGFP-treated cells was calculated as above to determine knockdown efficiency.

Transfection of hMSCs with siRNA against BCL2L2

Passage 3 untransduced hMSCs were seeded in 12-well-plates at a density of 10000/ cm^2 in complete expansion medium and allowed to adhere overnight. Transfection was carried out as described above, with the top polymer from siGFP screenings used at a 150 w/w ratio to complex with siRNA against BCL2L2 (siBCL2L2). A control with polymer at 150 w/w and

scrRNA was also prepared. After 10 minutes of incubation for nanoparticle self-assembly, the resulting nanoparticles were added to cells in complete expansion medium for a final siRNA dose of 60 *nM* in each well and allowed to incubate at 37°C for 4 hours. The media and nanoparticles were then removed and replaced with osteogenic medium. We also included one group of cells that were not transfected but were cultured under osteogenic conditions and another group of cells cultured under growth conditions as negative controls. The medium was replaced every 3–4 days and the cells were analyzed after 4 weeks by Alizarin Red S staining, calcium assay (Calcium Liquicolor Kit, Stanbio, Boerne, TX), and DNA assay (Quant-it PicoGreen dsDNA Assay Kit, Invitrogen) to ensure the results of the calcium assay were not due to differences in cell number between groups. Wells were transfected at *n* = 9, with *n* = 3 for each of the analyses.

Statistics

Unless otherwise stated, all results are presented as mean $\pm$ standard error of the mean. Statistical significance compared to the controls was assessed with a one-way ANOVA with post hoc Dunnett test, with *p* < 0.05 considered significant.

Results and Discussion

hMSC multilineage differentiation

Both GFP-transduced and untransduced hMSCs were subjected to osteogenic and adipogenic conditions for 4 weeks. Osteogenic and adipogenic groups were then stained with Alizarin Red S and Oil Red O, respectively. Transduced hMSCs retained the ability to differentiate down both osteogenic and adipogenic lineages at levels comparable to that of untransduced hMSCs (Supplementary Fig. S2). Hence, GFP⁺ hMSCs were deemed a valid model to assess the characteristics of non-viral transfection of hMSCs over the course of this experiment.

Polymer Synthesis

The number-averaged (*M_n*) and weight-averaged (*M_w*) molecular weights of all synthesized polymers are summarized in Table 1. The number of subunits per polymer chain was calculated using the *M_n*, with an average of 14–15 subunits. NMR analysis clearly showed the presence of acrylate peaks in the base polymer, which are no longer detectable after 1 hour end-capping reaction at room temperature (Supplemental Fig. S3). The reaction of E10 with the base polymer did not go to completion after only 1 hour, so further incubation at room temperature was required. This additional incubation time could lead to a side reaction (amide formation due to nucleophilic attack by the free end-capping amine on the base polymer's ester linkages), indicated by any additional peak in the product around 3–3.5 *ppm* [33]. Since this amide peak was not apparent in the spectrum, our product was not contaminated by this side product.

Nanoparticle Tracking Analysis (NTA)

Using NTA, individual nanoparticles suspended in a buffer of physiological salinity and pH were measured to calculate number-weighted average size and concentration (Fig. 2). As has been reported in previous studies [23, 24, 34], most nucleic acid particles formed with PBAEs are between 80–150 *nm* in diameter, a size range found to be amenable for cellular uptake [34]. By using NTA rather than dynamic light scattering (DLS), we were able to measure individual particles, including number-weighted size distributions and absolute particle concentration [36]. Increased polymer content tended to increase the number of particles formed with the same amount of siRNA, indicating that complexation of siRNA into nanoparticles was more efficient with more polymer. Interestingly, 4310-based particles

tend to be larger in size compared to 437- and 433-based particles; these also showed significant differences in transfection efficiency (see below). We therefore concluded that the representative end-modified PBAs measured by NTA (453, 447, 4410, 433, 437, and 4310) are good candidate materials for packaging siRNA.

siGFP transfection

We evaluated degradable polymers to choose an optimal polymer for siRNA delivery to hMSCs, assaying delivery efficacy by looking at GFP knockdown over time (Fig. 3). Based on previous results in another cell type [24], we included many cystamine (E10) terminated polymers in our study, along with polymers that have previously been seen to be effective in DNA delivery by our group and others [11, 22, 25, 37]. Although RNAi has been found to be an important potential tool in directing stem cell differentiation and MSCs are one of the most commonly studied cell types in differentiation studies [38], hMSCs have generally been found to be much harder to transfect than are other cells, such as HUVECs. This trend has been seen in DNA delivery studies [25], especially when the transfection is performed in the presence of serum proteins, as we have done here (10% v/v serum).

Through our polymer library approach, we were able to discover several polymers that caused effective knockdown. This research reveals several trends; many types of PBAs induced knockdown, but only a few were significantly better than the leading commercially available controls (Fig. 3). Interestingly, the subset of E10-terminated polymers examined here tended to cause relatively high knockdown compared to non-E10- or cystamine-terminated polymers. A major exception to this trend is 447 at high w/w ratios, which we have previously identified as one of the top-performing PBAs and which can be used for nucleic acid delivery to endothelial cells [24]. The other exception is 546, which, while very effective at high w/w, was also very toxic. The top E10-polymers, on the other hand, tended to be less toxic and were also effective at lower w/w. Certain base polymers, like B4-S3, showed little or no delivery efficacy with most end-capping amines, but were particularly effective when end-capped with E10, suggesting that the cystamine moiety itself may play an important role in siRNA delivery.

In particular, polymer 4310 formed successful nanoparticles, able to knock GFP levels down by $74\% \pm 6\%$ at 200 w/w and $72\% \pm 5\%$ at 150 w/w according to flow cytometry 12 days after transfection (Fig. 4). Both of these were significantly superior to Lipofectamine™ 2000 ($p < 0.01$). It should be noted that the flow cytometry data shown here are expected to be a slight underestimate. As seen in Supplementary Figure S1, the GFP⁺ hMSCs used were so brightly fluorescent that they were at the upper limit of sensitivity for the flow cytometer, even when using a 90% attenuation filter; therefore, small levels of GFP fluorescence knockdown in the brightest population of GFP⁺ cells could be underreported.

We examined the influence of cystamine further by directly comparing 4310 with 433 and 437 to test our hypothesis that the cystamine end-cap was the reason for 4310's efficacy. We also compared transfection with Lipofectamine™ 2000 and with naked siRNA as a baseline control (Fig. 5). 4310 outperformed Lipofectamine™ 2000, 433, and 437 in both magnitude and duration of knockdown, achieving knockdown of ~91% at 20 days post-transfection compared to ~50% using Lipofectamine™ 2000. This is in contrast to other work in siRNA-mediated knockdown using other delivery methods, including naked siRNA and lipid-based vehicles, in which knockdown duration was reported as 15 days or less [39–42]. Even after 20 days, 4310-mediated siRNA knockdown was only beginning to decline. Based on knockdown data from flow cytometry and the plate reader, 4310 at 150 w/w, which was not significantly different from 200 w/w, was used for later differentiation studies.

E10 (cystamine)-mediated siRNA release

The base polymer B4-S3 shows great difference in siRNA binding ability depending on its end-capping amine group (Fig. 6). 4310 is able to bind more tightly than is 433 at lower weight ratios upon initial complexation, which may explain why it is effective at lower w/w compared to other PBAEs in our study. The addition of reducing agent glutathione (GSH), however, has no apparent effect on 433-siRNA complexes, while 4310-siRNA complexes show complete release immediately after addition of GSH. Because intracellular GSH concentration is approximately three orders of magnitude higher than extracellular GSH concentration [43], this polymer's sensitivity to GSH could prove very useful for quick and efficient environmentally-triggered intracellular siRNA delivery once the polymeric nanoparticles enter the cytoplasm.

It is known that PBAEs are able to facilitate high cellular uptake (due to their net positive charge) as well as endosomal escape via the proton sponge mechanism because of titratable tertiary amines in the polymer backbone [44]. Just as insufficient release of DNA plasmids can have an inhibitory effect on their transcription and function [45, 46], we also expect that efficient release is important for optimal delivery of siRNA. As such, polymers like 4310 and other E10-terminated polymers may serve the dual purpose of strengthening the initial binding interaction between polymer and siRNA due to the presence of additional terminal primary amines as well as facilitating triggered release in the cytoplasm after cellular uptake due to the presence of disulfide linkages adjacent to these terminal primary amines.

While end-modification with E10 has proven effective for siRNA delivery and more than sufficient for effective knockdown in this system, other end-caps, such as E7, have been shown here and in previous work to facilitate highly effective delivery of both siRNA and also other cargo like DNA; we have thus far not shown E10-polymers to have increased efficacy in the delivery of DNA [24, 33], and further work may reveal other mechanistic details causing this difference. Future studies based on this work could explore other reducible structures and functional end-groups that could further increase the diversity of the polymer library and delivery efficacy.

siBCL2L2 transfection and its effect on osteogenesis

To ensure that the knockdown of GFP as a reporter would translate to successful knockdown when using functional genes, we tested our delivery system on hMSC osteogenic differentiation. siBCL2L2 was delivered to hMSCs and, after 4 weeks of cultivation under osteogenic conditions, osteogenesis was evaluated. Alizarin Red S staining (Fig. 7A–D) revealed that cells transfected with siBCL2L2 stained more intensely than did both cells transfected with scrRNA and untransfected cells, demonstrating increased osteogenic differentiation. As expected, cells cultured under standard growth conditions, rather than differentiation conditions, did not stain under Alizarin Red S. We also assayed the calcium content in each group to evaluate osteogenesis further to discover that the Alizarin Red S findings correlated with quantitative analysis of calcium content. Cells transfected with siBCL2L2 showed statistically higher calcium relative to all other groups (Fig. 7E). These results were not due to differences in cell number, as indicated by the results of the DNA assay, which revealed no significant differences in cell number between groups (Fig. 7F).

Our results indicate that 4310-mediated knockdown of BCL2L2 is capable of inducing a functional outcome in hMSCs, enhancing osteogenesis over the course of 4 weeks. While further optimization and transfection of other functional genes in addition to BCL2L2 may be needed to reach clinical application, this study demonstrates a proof-of-principle using our environmentally-triggered bio-reducible nanoparticle siRNA-delivery system. Critically, we have shown that cystamine-terminated poly(β -amino ester)s are effective for siRNA

delivery to hMSCs, especially when used at a high weight ratio of polymer to siRNA. This result is supported by studies in the literature that report the use of reducible polymers for siRNA delivery, albeit in the absence of serum [47] or at higher dosage and with lower efficacy [48, 49]. Here, we were able to not only deliver siRNA-loaded nanoparticles in medium with 10% serum and induce high knockdown (>70%), but also isolate the effects of the cystamine end-group from other effects due to the rest of the polymer. That the polymer's terminal group has such a dominant effect on the properties of PBAEs, which are linear polymers with 10–20 subunits along the base polymer chain, is unexpected but emphasizes the importance of small changes to the molecular structure of PBAEs, a feature that our library-based approach is well-suited to address.

Previous work by Andersen et al. [42] proposed the idea of using siRNA-functionalized nanoparticles combined with scaffolds to apply spatial control to MSC differentiation, thereby creating tissue grafts comprised of multiple tissue types. Much work has already been done in identifying target genes that, when knocked down via siRNA or miRNA, would enhance or even drive the differentiation of stem cells down various lineages (for a comprehensive review, see [38]). In order to use this data to its greatest potential, appropriate delivery biomaterials must first be identified, as most attempts to deliver siRNA non-virally to hMSCs have yielded poor results or required harsh conditions that compromise the viability of the cells being studied [50]. The polymeric nanoparticles we present here enable future translational studies that require safe and effective siRNA delivery to hMSCs. As can be seen here, the use of synthetic polymers allows us a high degree of flexibility in chemical and biophysical properties, which can be tuned for particular purposes. The approach used for our biomaterials could also be conducive to further functionalization of scaffolds and surfaces. For instance, numerous reactive groups on our polymers and the monomers that compose them could be used to modify scaffolds covalently or physically. While other researchers have shown effective *in vivo* delivery of siRNA using reducible polymers [49], the *in vitro* results reported here show greater nanoparticle stability and efficacy in serum as well as greater knockdown effects even when using a hard-to-transfect human stem cell type, suggesting that this polymer-based system may transition well in the future to a 3D model or an *in vivo* model. Moreover, the long knockdown duration we observed here, which has not been previously shown to our knowledge, may prove useful for long-lasting differentiation studies in which a certain level of gene expression is desired over the course of several weeks. The delivery strategy we present here could therefore enhance traditional differentiation-medium-based approaches to create complex grafts [3, 51]. Further work is warranted to understand specific mechanisms of delivery of siRNA using cystamine, related reducible moieties, and other degradable PBAEs.

Conclusion

We have synthesized and evaluated an array of polymers to find candidates that self assemble with siRNA to form nanoparticles with high knockdown to hard-to-transfect hMSCs. Knockdown in hMSCs of 91% at 20 days post-transfection was shown. Importantly, we have identified that a particular polymer terminal group, cystamine, facilitates especially effective siRNA delivery to hMSCs. Finally, we validated this polymeric siRNA delivery system as a potential method to aid differentiation of hMSCs by showing that 4310-mediated knockdown of BCL2L2 causes enhanced osteogenesis of hMSCs compared to controls.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Polymer synthesis reaction and monomer structures. One backbone (B) and one sidechain (S) monomer are mixed to form an alternating copolymer via Michael addition. The resulting acrylate-terminated base polymer is functionalized with one end-cap (E) monomer.

Fig. 2. Nanoparticle Tracking Analysis (NTA). Number-averaged mean and mode particle size for all tested polymers was between 80–150 nm. Nanoparticle concentration was $0.5 - 2 \times 10^9$ /mL.

Fig. 3. Nanoparticle induced knockdown and relative metabolic activity. (A) eGFP signal from cells transfected with siRNA was normalized to the signal from cells transfected with scrRNA and subtracted from 100% to calculate knockdown. Knockdown data are from 12 days post-transfection and * $p < 0.01$. (B) Relative metabolic activity of nanoparticle-treated samples compared to untreated control. Metabolic activity measurements are from 24 hours post-transfection.

Fig. 4. Median GFP intensity per cell, measured by flow cytometry 12 days after transfection with 4310, showed knockdown significantly superior to Lipofectamine™ 2000 transfection (**p < 0.01).

Fig. 5. Comparison between 4310 and other vehicles of siRNA delivery. (A) At a weight ratio of 150 w/w, 433, 437, and naked siRNA induced little to no knockdown in contrast to Lipofectamine™ 2000 and 4310. 4310, in turn, outperformed Lipofectamine™ 2000, with robust knockdown still evident 20 days post-transfection. Fluorescence microscopy (B–I) supported observations from the plate reader. Scalebar: 200 μ m.

Fig. 6. PBAE/siRNA binding evaluation. PBAE was added to siRNA at varying weight ratios, mixed with either PBS alone or PBS with GSH (final 5 *mM* GSH concentration) and then added to the lanes of agarose gels. There is progressively tighter binding and greater retardation of siRNA in the gel with greater weight ratios. 4310-based nanoparticles release siRNA in the presence of GSH, whereas 433-based nanoparticles do not release siRNA in the presence of GSH.

Fig. 7. Quantification of osteogenesis and cell number. Alizarin Red S staining showed noticeably denser staining from cells given (A) siBCL2L2 + OM compared to cells given (B) scrRNA + OM and (C) cells given only OM. (D) Cells given EM only did not stain under Alizarin Red S. (E) Quantitatively, the siBCL2L2 + OM group produced more calcium than did the scrRNA + OM group ($32\% \pm 9\%$ more calcium, $*p < 0.05$) and the OM only group ($52\% \pm 10\%$ more calcium, $**p < 0.01$). No significant difference was found in calcium content between the scrRNA + OM and OM only groups. (F) A DNA assay on replicate samples showed no differences in cell number between groups (one-way ANOVA $p > 0.05$), ruling

out increased cell density as a cause for the increased calcium content. OM = osteogenic medium, EM = expansion medium. Scalebar: 200 μm .

Table 1

Polymer molecular weight and polydispersity index (PDI) measured by GPC

Polymer	Mn (Da)	Mw (Da)	PDI	# subunits per chain
B4-S3-E3	4295	6805	1.584	17
B4-S3-E7	4288	6891	1.607	17
B4-S4-E7	4940	10176	2.060	18
B4-S5-E3	4426	7459	1.685	15
B4-S5-E4	4991	11919	2.388	17
B4-S5-E6	4840	8080	1.669	17
B4-S5-E7	4262	7181	1.685	15
B4-S5-E8	5464	12365	2.263	19
B5-S4-E4	4183	7637	1.826	14
B5-S4-E6	4381	7912	1.806	15
B6-S3-E3	4243	7090	1.671	15
B6-S3-E6	4206	7806	1.856	15
B3-S4-E10	3527	6599	1.871	14
B3-S5-E10	3096	5173	1.671	11
B3-S6-E10	3257	5258	1.614/	11
B4-S3-E10	4035	7896	1.957	16
B4-S4-E10	3937	7551	1.918	14
B4-S5-E10	4774	7252	1.519	16
B5-S3-E10	3099	4637	1.496	11
B5-S4-E10	3677	5793	1.575	13
B5-S5-E10	4108	6140	1.495	13
B6-S3-E10	4198	7125N	1.697	14