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Cationic Hyperbranched Polymers with Biocompatible Shells for siRNA Delivery

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Abstract

Cationic hyperbranched polymers (HBP) were prepared by self-condensing vinyl polymerization of an atom transfer radical polymerization (ATRP) inimer containing a quaternary ammonium group. Two types of biocompatible shells, poly(oligoethylene glycol) methacrylate (polyOEGMA) and poly(2-(methylsulfinyl) ethyl methacrylate) (polyDMSO), were grafted respectively from HBP core to form core-shell structures with low molecular weight dispersity and high biocompatibility, polyOEGMA-HBP and polyDMSO-HBP. Both of the structures showed low cytotoxicity and good siRNA complexing ability. The efficacy of gene silencing against Runt-related transcription factor 2 (*Runx2*) expression and the long-term assessment of mineralized nodule formation in osteoblast cultures were evaluated. The biocompatible core-shell structures were crucial to minimizing undesired cytotoxicity and nonspecific gene suppression. polyDMSO-HBP showed higher efficacy of forming polyplexes than polyOEGMA-HBP due to shell with lower steric hindrance. Overall, the gene silencing efficiency of both core-shell structures was

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Author Contributions

S.L. and M.O. contributed equally to this work.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biomac.8b00902. Details of calculations of degree of branching, cytotoxicity, and complexations of the hyperbranched polymers, complexations, agarose gel images of core-shell structures, and Alizarin red staining results of cells treated with *Runx2* siRNA alone without codelivery cationic polymers (PDF)

comparable to commercial agent *Lipofectamine*, indicating long-term potential for gene silencing to treat heterotopic ossification (HO).

Graphical Abstract

INTRODUCTION

RNA interference (RNAi) is the process of post-transcriptional silencing of gene expressions triggered by short interfering RNA (siRNA). The therapeutic potential of RNAi has been far-reaching since it can prevent the production of targeted proteins that could lead to diseases, including inherited disorders, some types of cancers and certain viral infections.^{1–9} For example, heterotopic ossification (HO) is of an urgent clinical concern, it may occur as a consequence of musculoskeletal trauma from blast and high-energy injuries, total joint arthroplasty (TJA), traumatic brain injury, or spinal cord injury.^{10–12} Contemporary treatments of HO such as anti-inflammatory drugs and radiation therapy have adverse effects and are not inherently designed to correct the molecular mechanism of the etiology of HO. It was suggested the cause of HO may be due to dysregulated signals in the bone morphogenetic protein osteogenic cascade. siRNA delivery against Runt-related transcription factor 2 (*Runx2*) genes can potentially decrease mRNA expression, inhibit activity of the osteogenic marker alkaline phosphatase (ALP) and thus abrogate HO.¹³ However, the selective delivery of siRNA into cells has been a challenge since unmodified siRNA that is unstable in bloodstream can be immunogenic and difficult to pass through cell membranes.^{2,14} There are two major types of siRNA carriers to facilitate the delivery, viral and nonviral systems.^{15,16} The viral system is risky due to potential virus replication and inflammation reactions. Therefore, development of efficient nonviral carriers based on cationic lipids or synthetic polymers has become a more promising option.^{15,17–28}

Positively charged polymers can generate polyplexes with negatively charged siRNA and thus improve the transfection of siRNA into cells via adsorptive endocytosis.^{29,30} Some cationic polymeric materials include chitosan,²⁴ cationic polypeptides,³¹ cationic lipids,³² polyethylenimine (PEI),^{33,34} quaternized or nonquaternized poly(2-(dimethylamino)ethyl methacrylate) (polyDMAEMA)^{35,36} poly(meth)acrylates with tertiary sulfonium groups,³⁷ and so forth. However, polymers with the “naked” positive charges on the surface can cause toxic effect to cell survival and nonspecific gene suppression. Such surface charges can be “screened” by incorporating biocompatible segments of poly(ethylene glycol) (PEG) or poly(oligo(ethylene glycol) methacrylate) (polyOEGMA) via “PEGylation”.^{38–41} These biocompatible segments can be introduced in a block copolymer structure that contains PEG or polyOEGMA as a second block but can still interact with siRNA to form biocompatible

micelles under heterogeneous conditions.^{42,43} Another approach is to use multicomponent cationic nanogels prepared from emulsion, which shows higher structural stability than micelles. As an example, a biocompatible nanogel made of quaternized DMAEMA, OEGMA, and cross-linkers was previously synthesized in microemulsion.⁴⁴ It showed promise for both plasmid DNA (pDNA) and siRNA delivery.⁴⁴ However, the preparation of either micelles or multicomponent nanogels employs self-assembly or polymerization under heterogeneous conditions and requires extensive postpurifications.⁴⁵ Alternatively, biocompatible cationic polymeric carriers can be prepared under homogeneous conditions in the form of dendritic polymers, such as multiarm polymers,⁴⁶ hyperbranched polymers (HBP),^{47–50} and starlike polymers with a cationic cross-linked core.^{17,36}

Hyperbranched polymers (HBP) possess interesting properties such as abundance of intramolecular cavities, high-surface functionality, high solubility, and unique viscosity, compared to their linear analogues.^{51–54} Because of these properties, HBP can have much higher transfection efficiency than their linear analogues with a similar molecular weight.^{46,55–59} Hyperbranched cationic polymers can be easily synthesized by self-condensing vinyl polymerization (SCVP) using controlled radical polymerization on a large scale.^{60–65} However, due to combination of step-growth and chain-growth mechanisms HBP with higher degree of branching (DB) can only be achieved at the cost of broad molecular weight distribution. Recently, it was demonstrated that synthesis of HBP in microemulsion provides polymers with much lower dispersity.⁶⁶ Yet, to prepare HBP with low dispersity under homogeneous conditions remains to be an unsolved problem.

Another challenge for successful siRNA delivery is the choice of biocompatible segments. Recent reports of immunogenicity issues of PEG-based polymers have come to light primarily due to the abundance of modern drugs and cosmetics which employ PEG or polyOEGMA as a part of their formulation.⁶⁷ Moreover, if OEGMA is densely grafted to the core it may sterically hinder the complexation with siRNA and diminish the efficiency of gene silencing. Thus, development of new biocompatible polymers for siRNA delivery is welcomed.^{67,68} Recently, the synthesis of a highly biocompatible polymeric analogue of dimethyl sulfoxide (DMSO) (poly(2-(methylsulfinyl) ethyl acrylate)) by atom transfer radical polymerization (ATRP) was reported.⁶⁹ The polymer showed very high hydrophilicity and also much smaller steric hindrance than OEGMA.^{70–72} Therefore, the efficiency of this new type of polymer as protective layers and comparison to polyOEGMA for siRNA complexation and delivery need to be investigated.

Herein, we report a direct synthesis of cationic HBP with low molecular weight dispersity via activator generated by electron transfer (AGET) ATRP.^{61,73–75} SCVP of a cationic inimer bearing a quaternary ammonium group was successfully conducted. The degree of branching (DB) was adjusted (16%, 22%, and 34%) by changing the ratio of activator to deactivator in an AGET ATRP process. Because of the use of charged inimers, the charge–charge repulsion between each growing chain reduced the oligomer coupling and generated HBPs with relatively low dispersity (<1.8) even at DB of 34%. To increase the biocompatibility and decrease the toxic effect of the cationic HBP core, a biocompatible shell layer based on either polyOEGMA or poly(2-(methylsulfinyl) ethyl methacrylate) (abbreviated as polyDMSO) was introduced via surface initiation polymerization of the

corresponding monomers from the cationic core using activator regenerated by electron transfer (ARGET) ATRP. Both of the core-shell structures had low cytotoxicity down to 1 mg/mL. Because of the lower steric hindrance, the polyDMSO-based HBP showed higher complexing efficiency with siRNA. The long-term efficacy of cationic polymer-mediated RNAi attack on *Runx2* expression in wild-type osteoblasts was determined by using quantitative real-time polymerase chain reaction (PCR) detection. The assessment of mineralized nodule formation in osteoblast cultures was conducted by the Alizarin Red S staining. While the naked HBP core showed nonspecific gene suppression due to cytotoxicity, the biocompatible core-shell structures were crucial to minimizing undesired cytotoxicity and nonspecific gene suppression. polyDMSO-HBP showed higher efficacy of forming polyplexes with siRNA than the polyDMSO-HBP due to lower steric hindrance of the polyDMSO shell. Interestingly, the gene silencing efficacy of both polyOEGMA-HBP and polyDMSO-HBP was similar and comparable to *Lipofectamine*. The results indicated that the core-shell polymer strategy based on a cationic HBP core may have long-term potential for treatment of HO without any undesirable cytotoxic effects.

EXPERIMENTAL SECTION

Materials.

2-Bromoisobutyric acid (98%), 2-bromoethanol (95%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%), poly-(ethylene glycol) methyl ether methacrylate (OEGMA, MW = 500), methacrylic acid (99%), *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 99%), *N,N*-dimethylamino pyridine (DMAP, 99%), copper bromide (99.99%), ascorbic acid, (AA, 99%) were purchased from Sigma-Aldrich. 2-(Methylthio)-ethanol (99%) was purchased from Alfa Aesar. Hydrogen peroxide solution (30%) was purchased from Fisher Scientific. All methacrylate monomers were passed through basic alumina columns before use. Tris(2-pyridylmethyl)amine (TPMA) was synthesized according to previous procedures.⁷⁶ All solvents and other chemicals were of reagent quality and were used as received unless special treatments discussed below were applied.

Instrumentation.

¹H NMR measurements were performed on a Bruker Avance 300 MHz spectrometer. Molecular weight and molecular weight distribution (dispersity, $\bar{M}_w/\bar{M}_n$) were determined by size exclusion chromatography (SEC) equipped with a Waters 515 HPLC pump, Wyatt Optilab refractive index detector, Wyatt DAWN HELEOS-II multiangle light scattering detector and PSS GRAM columns containing polyester copolymer networks at 50 °C. The cationic hyperbranched polymers were analyzed in pure DMF phase at flow rate of 1 mL/min. The core-shell structures were analyzed in 50 mM LiBr DMF solution as eluent phase at flow rate of 1 mL/min.

Synthesis of Cationic ATRP Inimer.

2-Bromoethyl α -bromoisobutyrate was synthesized by esterification between 2-bromoethanol and 2-bromoisobutyric acid. In a typical procedure, 3.4 g of 2-bromoethanol (1 equiv), 5 g of 2-bromoisobutyric acid (1.1 equiv), 6.38 g of EDC (1.5 equiv), and 0.17 g

of DMAP (0.05 equiv) were dissolved in 50 mL of dichloromethane in a 100 mL round-bottom flask equipped with a stirring bar in an ice bath. The solution was purged with N₂ for 10 min and allowed to warm up to room temperature. After 24 h, the solution was washed with 50 mL 1 M HCl solution, saturated NaHCO₃ solution and brine 3 times each. The organic phase was collected and dried over anhydrous MgSO₄. The solvent was removed under vacuum to yield yellow liquid product (yield 85%). ¹H NMR (300 MHz, CDCl₃): δ (ppm) = 4.48 (1H, t, COOCH₂CH₂Br), 3.55 (2H, t, COOCH₂CH₂Br), 1.98 (6H, s, Br(CH₃)₂CCOO). The cationic inimer was synthesized by a subsequent quaternization (Menschutkin reaction). In a typical procedure, 5 g of 2-bromoethyl α -bromoisobutyrate (1.05 equiv) and 2.73 g of DMAEMA (1 equiv) were dissolved in 25 mL of DMF in a 50 mL round-bottom flask equipped with a stirring bar at 35 °C. The solution was allowed to react for 2 h. The solution was then precipitated in 500 mL of diethyl ether. The precipitate was collected and dried under vacuum to give white solid (98%). ¹H NMR (300 MHz, DMSO-*d*₆): δ (ppm) = 6.09 (1H, s, CHH=CCH₃), 5.78 (1H, s, CHH=CCH₃), 4.58 (4H, m, COOCH₂CH₂N-(CH₃)₂CH₂CH₂OCO), 3.84 (4H, m, COOCH₂CH₂N-(CH₃)₂CH₂CH₂OCO), 3.20 (6H, s, COOCH₂CH₂N-(CH₃)₂CH₂CH₂OCO), 1.93 (6H, s, Br(CH₃)₂CCOO), 1.91 (3H, s, CH₂=CCH₃).

Synthesis of Sulfoxide Containing Monomer 2-(methylsulfinyl)ethyl methacrylate (MSEMA).

2-(Methylthio)-ethyl methacrylate (MTEMA) was synthesized according to our previously reported procedure.³⁷ In a typical oxidation procedure, 25.5 g of MTEMA was added to a 100 mL round-bottom flask sealed with rubber stopper. The flask was kept in an ice bath and purged with N₂; then 18.3 g of hydrogen peroxide solution (20%) was slowly injected into the flask at rate of 50 μ L/min. The reaction was allowed to stir for 24 h and was then stopped by adding 50 mL of deionized water. The aqueous solution was washed 3 times with 100 mL of dichloromethane. The organic phase was collected and dried over magnesium sulfate. Solvent was removed under vacuum to give white solid (yield 75%). ¹H NMR (300 MHz, DMSO-*d*₆): δ (ppm) = 6.05 (1H, s, CHH=CCH₃), 5.71 (1H, s, CHH=CCH₃), 4.35–4.54 (2H, m, C=OOCH₂CH₂), 3.95–3.23 (2H, m, CH₂CH₂S=O), 2.60 (3H, s, S=OCH₃), 1.89 (3H, s, CH₂=CCH₃).

Synthesis of Cationic Hyperbranched Polymers (HBP).

The synthesis employed an AGET ATRP. In a typical procedure, 1 g of cationic inimer (50 equiv), 0.041 g of CuBr₂ (4 equiv), and 0.081 g of TPMA (6 equiv) were dissolved in 1.4 mL of DMSO and 0.1 mL of DMF in a 10 mL Schlenk flask equipped with a stirring bar. The solution was degassed by three cycles of freeze–pump–thaw and then filled with N₂. The flask was then placed in 35 °C oil bath. A 0.011 g sample of ascorbic acid (1.32 equiv) in 0.1 mL of DMSO was injected into the flask under N₂ purge. The reaction was stopped after 24 h. The hyperbranched polymer was purified by dialysis (MWCO = 100–500 Da) against water and dried under vacuum. The conversion was measured by ¹H NMR and the molecular weight and dispersity were measured by SEC-MALS. The calculation of degree of branching (DB) is elaborated in the Supporting Information. A similar approach compared to a previous report was used.⁶⁶ In general, to determine the DB polymers were purified by dialysis and analyzed by ¹H NMR. The DB was calculated under the assumption that there is minimal radical termination and intramolecular cycling. The number of newly

formed initiating sites should equal to the reacted initiating sites and the number of original initiating sites in the inimer should equal to the combined amount of reacted and unreacted vinyl groups. (Scheme S1) These two equations, combined with the integration of NMR peaks ($\delta = 2.6\text{--}2.8$, $0.7\text{--}2.2$, and $5.7\text{--}6.3$), allowed for the calculation of reactivity ratio $r = k_A^*/k_B^*$ and the DB.

Synthesis of Core–Shell Structure polyOEGMA–HBP.

The synthesis employed an ARGET ATRP. In a typical procedure, 2 g of OEGMA (100 equiv), 17.2 mg of cationic HBP (1 equiv. per Br), 5.3 mg of CuBr_2 (0.6 equiv), 9.3 mg of TPMA (0.8 equiv) were dissolved in 7.9 mL DMSO and 0.25 mL DMF in a 25 mL Schlenk flask equipped with a stirring bar. The flask was degassed by three cycles of freeze–pump–thaw and filled with nitrogen after the last cycle. 14.1 mg ascorbic acid (2 equiv) dissolved in 0.1 mL of DMSO was injected into the solution under nitrogen purge. The mixture was allowed to react on a 40 °C heating plate. An initial sample ($t = 0$) was collected by syringe. Samples were taken periodically to measure conversion via ^1H NMR and molecular weight via SEC-MALS. The polymer was purified by dialysis (MWCO = 100–500 Da) against water and dried by lyophilization.

Synthesis of Core–Shell Structure polyDMSO–HBP.

The synthesis employed an ARGET ATRP. In a typical procedure, 0.5 g of 2-(methylsulfinyl)ethyl methacrylate (MSEMA) (100 equiv), 12.2 mg of cationic hyperbranched polymer (1 equiv. per Br), 3.8 mg of CuBr_2 (0.6 equiv), 4.9 mg of TPMA (0.8 equiv) were dissolved in 3.65 mL of DMSO and 0.1 mL of DMF in a 10 mL Schlenk flask equipped with a stirring bar. The flask was degassed by three cycles of freeze–pump–thaw and filled with nitrogen after the last cycle. Ten milligrams of ascorbic acid (2 equiv) dissolved in 0.1 mL of DMSO was injected into the solution under nitrogen purge. The mixture was allowed to react in a 40 °C heating plate. An initial sample ($t = 0$) was collected by syringe. Samples were taken periodically to measure conversion via ^1H NMR and molecular weight via SEC-MALS. The polymer was purified by dialysis (MWCO = 100–500 Da) against water and dried by lyophilization.

Cytotoxicity Tests with SH-SY5Y Cells.

SH-SY5Y epithelial neuroblastoma cells (ATCC CRL-2266) were cultured in a base medium composed of a 1/1 ratio of Eagle's Minimum Essential Medium and F-12 growth medium. Fetal bovine serum (FBS) was added to base medium to reach 10% final concentration of FBS, as per cell line manufacturer's instructions (GenTarget Inc., cat. no. SC042). Before testing, cells were grown on the above medium in Corning T-75 culture flasks at 37 °C and 5% CO_2 in a humidified incubator. Prior to biocompatibility testing, each well of a 96-well culture plate was seeded with ~10,000 cells in 150 μL of growth medium. After 24 h of incubation, tested polymers were added to wells in concentrations of 1000, 333, 111, 37, 12, 4, 1.4, and 0.5 $\mu\text{g/mL}$ via serial dilutions repeated in triplicate. Untreated + control wells contained cells and media only, and – control wells were treated with 1% Triton X to kill all cells. After 48 h of exposure to test polymer, 100 μL was taken from each test well and combined with 100 μL ATP assay solution (CellTiter-Glo Luminescent Cell Viability Assay from Promega) in a prewarmed black 96-well reading plate, then incubated

for 30 min at 23 °C. End point luminescence was measured for each well as an average of 5 readings per well, using a BioTek plate reader with Gen5.2.09 software. Percent survival was approximated as averaged triplicate luminance of treated versus control wells; variance in luminance across experimental and control replicates were reported as error.

siRNA Complexation Using Negative Control siRNA.

To determine each polymer's efficiency of complexing with siRNA, agarose gel shift assays were conducted for the polymers. Negative control siRNA from IDT DNA was used in the experiment. Since unbounded siRNA and bounded siRNA possess opposite charge, the gel shift assay indicates the weight ratios at which siRNA is incorporated into a given complex. Polyplexes were prepared by incubating polymers of varying concentrations (diluted with nuclease-free ultrapure water) with 500 ng of siRNA to produce polymer/siRNA weight ratios of 400/1, 200/1, 100/1, 80/1, 60/1, 40/1, 20/1, 10/1, 4/1, 0.8/1, 0.16/1, and 0/1 (0/1 are control wells containing 500 ng of siRNA and water alone). Upon addition of siRNA, polyplexes were incubated at 23 °C for 60 min, after which they were weighed with 5 μ L of nuclease-free glycerin for total loading volumes of 30 μ L per well. Polyplexes were then loaded into 100 mL volume 2% agarose gels prepared with Tris/Borate/EDTA (TBE) buffer and 7.5 μ L of ethidium bromide. After loading, electrophoresis was conducted under 100 V current for 60 min. After electrophoresis, all gels were photographed with 500 ms exposures under ultraviolet illumination via Labnet's ENDURO GDS system. To determine the stability of siRNA polyplexes, negative control siRNA (naked siRNA) and siRNA polyplexes at polymer/siRNA weight ratio of 400/1 were incubated in alpha-minimum essential medium (α MEM) supplemented with 10% FBS at 37 °C. The samples were collected at different time points (4, 12, 24, 36, and 48 h) and analyzed by agarose gel electrophoresis.

Wild-Type Osteoblasts Cell Proliferation Assay.

Wild-type osteoblasts were isolated from newborn mouse calvaria and cultured in alpha-minimum essential medium (α MEM) supplemented with 10% fetal bovine serum (Denville Scientific) and 1% penicillin/streptomycin (Invitrogen). All cells were used for experiments before passage 4. To determine cell counts, osteoblasts were seeded in 24-well plates (1.0×10^4 cells/well) and incubated with hyperbranched polymers. At day 1, 3 and 5, adherent cells were washed with PBS and incubated with trypsin at 37 °C for 5 min. After trypsinization, cell suspensions were transferred to a hemocytometer with Trypan Blue Solution and counted under the microscope.

Quantitative PCR.

Cells were seeded at 2.0×10^4 cells/well in 24-well plates and RNA was isolated using TRIzol reagent (Life Technologies). For quantitative real-time PCR analyses, 100 ng of total RNA was reverse-transcribed using Superscript first-strand synthesis system (Invitrogen) with Oligo (dT) as a primer. The levels of gene expressions were measured by quantitative real-time PCR using ABI Prism 7500 (Applied Biosystems). Taqman probes for *Runx2* (Mm 00501578_m1), *Osterix/Sp7(Osx)* (Mm 04209856_m1), alkaline phosphatase (*Alp*) (Mm00475831_m1), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)

(Mm99999915_g1) were used for quantification. Data were normalized to *Gapdh* expression using the comparative Ct method.

Alizarin Red S Staining.

Cells were seeded at 2.0×10^4 cells/well in 24-well plates and incubated with osteogenic differentiation medium containing 10 mM β -glycerophosphate and 50 μ g/mL L-ascorbic acid. After 14 days in culture, cells were fixed in 70% ethanol for 15 min at room temperature. Cells were stained with 40 mM alizarin red S (Sigma-Aldrich) for 10 min and then rinsed five times with DI water to minimize nonspecific staining. The stained area was digitally photographed and measured using of the ImageJ (National Institutes of Health, U.S.A.).

RNAi Experiments.

Silencer select Predesigned siRNA against mouse *Runx2* gene (5'-CAAGUGCGGUGCAAACUUUtt-3' and 5'-AAAGUUUGCACCGCACUUGtg-3') and scrambled siRNA (Silencer Negative Control #1 siRNA) were purchased from Ambion (Austin, TX). siRNA polyplexes were prepared by mixing hyperbranched polymers and siRNAs (20 pM) in polymer/siRNA weight ratios ranging from 5/1 to 20/1 in volumes up to 5 μ L. For transfection, 2.0×10^4 cells/well were seeded in 24-well plates the day before transfection. The siRNA polyplexes were delivered 24 h prior to delivery of recombinant human bone morphogenetic protein 2 (rhBMP-2, R&D Systems, 100 ng/mL). Cell culture media were refreshed in conjunction with RNAi treatments and rhBMP-2 every 2 days for the duration of the study.

Statistical Analysis.

All results were expressed as means $\pm$ standard deviation of triplicate measurements with all experiments independently repeated at least three times. Unpaired Student's t tests were used to evaluate statistical differences. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSIONS

Synthesis of Cationic Hyperbranched Polymers (HBPs).

An ATRP inimer bearing a quaternary ammonium group was synthesized by Menshutkin reaction between 2-bromoethyl α -bromoisobutyrate and DMAEMA in dimethylformamide (DMF), a polar aprotic solvent, at 35 $^{\circ}$ C.^{48,62,77} The reaction was completed within 2 h; the inimer yield was 98% after purification. Hyperbranched polymers were prepared via SCVP using AGET ATRP of this inimer with CuBr₂/tris(2-pyridylmethyl)amine (TPMA) complexes as the catalyst source and ascorbic acid as reducing agent to generate Cu^I species (Scheme 1).

Three different amounts of reducing agents were used: [cationic inimer]/[ascorbic acid]/[CuBr₂]/[TPMA] = 50/ x /4/6, where x = 0.3, 1.3, or 4.4. SCVPs were conducted at 35 $^{\circ}$ C in DMSO using 38 wt % of inimer (Table 1). After 1 h, conversion exceeded 95% in all polymerizations. By using different amount of reducing agent, the feed ratios of [Cu^I/TPMA] activator to [Cu^{II}/TPMA] deactivator was adjusted. By increasing the ratio of

ascorbic acid to CuBr_2 from 0.3 to 4.4, the DB decreased from 34% to 16%. This is because at lower concentration of reducing agent, and consequently higher concentration of deactivator, the deactivation rate was faster to allow both types of initiating sites to be activated at statistical ratio, leading to higher DB. Meanwhile, a lower concentration of deactivator facilitated chain propagation, that is, reaction of a radical with more vinyl groups, in one activation deactivation cycle, leading to low primary chain segment and low DB (Figure S1). The details of calculation of DB are included in Supporting Information.

Generally the formation of hyperbranched structures follows the step/chain-growth mechanism.⁷⁹ As a result, the polymerization is accompanied by random polymer–polymer coupling which leads to extremely broad molecular weight distribution.^{66,80} Recently, it was shown that confining growing polymers in droplets of microemulsions gives low molecular weight dispersity below 2. This is because growing chain-end of radicals in separate droplets have a diminished chance of random polymer–polymer coupling.⁶⁶ The molecular weights of the cationic HBPs were measured by SEC in pure DMF eluent phase without addition of any salt. SEC traces of all the polymers showed monomodal distribution, indicating relatively homogeneous structure. Interestingly, all the HBPs showed relatively narrow molecular weight distribution. Polymer with DB of 34% had molecular weight of 5700 and dispersity = 1.78 (Figure 1). With lowered DB, the molecular weight increased, indicating larger fraction of linear structural units. Such low molecular weight dispersity could be due to the charge–charge repulsion between each growing chain, which reduces the chance of oligomer coupling and favors the reaction of inimers with existing chains. With sufficient amount of Cu(II) species, the polymerization can still generate DB as high as 34%.

Synthesis of Biocompatible Core–Shell Structures.

As the surface of the HBPs contains abundant initiating bromine sites and cationic species, the cytotoxicity can be reduced by grafting biocompatible polymers from the surface of the HBP to form core–shell structures. In order to achieve high transfection efficiency,⁵⁵ HBP samples with the highest DB of 34% were selected for the subsequent synthesis of core–shell structures (Scheme 2). OEGMA as a common “PEGylation” agent has been widely used to improve biocompatibility in biofunctional polymers synthesized by controlled radical polymerizations. On the other hand, a novel biocompatible polymeric analogue of DMSO, poly(2-(methylsulfinyl)ethyl acrylate), has also been developed. This polymeric analogue of DMSO possesses high biocompatibility, high water-solubility, and smaller steric hindrance compared to polyOEGMA. In this work, we introduce a methacrylate version of the polymer based on monomer of 2-(methylsulfinyl)ethyl methacrylate (MSEMA). OEGMA (MW = 500) and MSEMA were grafted from the cationic HBP to form core–shell structures polyOEGMA–HBP and polyDMSO–HBP, respectively (Scheme 2). The conditions for polyOEGMA–HBP are $[\text{OEGMA}]/[\text{per Br}]/[\text{ascorbic acid}]/[\text{CuBr}_2]/[\text{TPMA}] = 100/1/2/0.6/0.8$ ($m/v = 1/4$ in DMSO at 40 °C). Three samples with different sizes of shell were synthesized. As shown in Table 2, core–shell structures polyOEGMA–HBP with shell DP of 5, 48, and 95 were synthesized. SEC traces showed that the core–shell structures had low molecular weight dispersity below 1.4 (Figure 3a). With the shell DP increased from 5 to 95, the molecular weight analyzed by SEC MALS increased from 31 500 to 1 500 000

(Figure 2). The synthesis conditions for polyDMSO–HBP are $[MSEMA]/[per\ Br]/[ascorbic\ acid]/[CuBr_2]/[TPMA] = 100/1/2/0.6/0.6$ ($m/v = 1/8$ in DMSO at 40 °C). Three samples with different shell DPs, that is, 5, 35, and 45 were synthesized at different conversions. The SEC curves showed that all the polymers have monomodal distribution. As the DP increased from 5 to 45, the molecular weight by SEC MALS increased from 15 900 to 62 100 with dispersity increased from 1.41 to 1.73 (Figure 2).

Biocompatibility and siRNA Complexation.

Since cell toxicity of siRNA delivery material is the major obstacle that limits the use of siRNA for therapeutic applications, cell toxicity of the polymers was first examined on SH-SY5Y epithelial neuroblastoma cells. To determine the siRNA complexation efficiency of the HBP and core–shell structures, agarose gel shift assays were conducted for the polymers. Negative control siRNA from IDT DNA was used in the experiment. The HBP with a DB of 34% showed a hydrodynamic volume-mean size of 3.37 nm and a zeta potential of +38.1 mV indicating high concentration of positive charges on the surface of HBP (Figure S2a). The HBP formed polyplexes with siRNA at a weight ratio of polymer/siRNA = 4/1 (Figure S2b). However, due to the surface charge the HBP became highly toxic upon concentration of 0.33 mg/mL (Figure S2c). By grafting a biocompatible shell from the cationic core, the hydrodynamic size of the core–shell structures increased while the positive surface charge decreased as expected. (Table 2) Core–shell structure polyOEGMA–HBP with a shell DP of 5 had a hydrodynamic size of 10.6 nm and a zeta potential of +10.1 mV. In comparison, polyDMSO–HBP with the same shell DP has a smaller size of 7.7 nm and a larger zeta potential of +12.7 mV. Crucially, both of the structures showed equally improved biocompatibility than the naked HBP core (Figure 3).

In agarose gel experiment, polyOEGMA–HBP with a shell DP of 5 was observed to form complexes with negative control siRNA at polymer/siRNA weight ratio of 20/1 and formed complete complexes at weight ratio of 400/1 (Figure 4a). As the shell DP increased to 48, the polymers did not bind to siRNA at weight ratio of polymer/siRNA lower than 200/1 and no complexes were formed up to weight ratio of 400/1 for polyOEGMA–HBP with shell DP of 95 (Figure S5). This observation indicated that lowering the steric hindrance of the biocompatible shell can increase the siRNA complexing efficiency. In comparison, polyDMSO–HBP with shell DP of 5 was observed to start to form complex with negative control siRNA at a weight ratio of 10/1 and fully formed complexes at 200/1 (Figure 4b). Moreover, for polyDMSO–HBP with shell DP of 45, complexes started to form at a weight ratio of 40/1 (5 times lower than polyOEGMA–HBP with same shell DP, Figure S5). Such observation indicated that due to the smaller steric hindrance and low cytotoxicity, polyDMSO can provide similar biocompatibility enhancement as polyOEGMA while with higher siRNA binding efficiency. Moreover, because the lower steric hindrance arises from the lower molecular weight of the polymer shell, the actual loading of cationic charges is also higher, which synergistically improved the complexing efficiency. The stability of siRNA polyplexes was assessed by agarose gel electrophoresis. (Figure S6) Interestingly, the results suggest that polyDMSO–HBP polyplexes are more stable than polyOEGMA polyplexes in culture media containing 10% FBS up to 2 days.

EFFECTS OF CATIONIC POLYMERS WITHOUT SIRNA ON OSTEOGENIC DIFFERENTIATION

We then examined the cytotoxicity of a series of different concentrations of HBP core polymers and core-shell structures on primary osteoblasts by cell counting. Figure 5a shows that cell proliferation was largely unaltered by 2.5 $\mu\text{g/mL}$ of hyperbranched polymer treatments after 5 days in culture, whereas 10 $\mu\text{g/mL}$ of the naked HBP core significantly decreased osteoblast number after 5 days (Figure 5b), suggesting the degree of cytotoxicity caused by the HBP core. To verify if cationic HBP core alone affect the gene expression in osteoblasts, cells were treated with each polymer without siRNA. Results indicated that all polymers at 2.5 $\mu\text{g/mL}$ had no significant effect on *Runx2*, *Osx*, and *Alp* expressions compared to untreated groups at 30 h (Figure 5c) and 7 days (Figure 5e) after the treatment. However, HBP core at 10 $\mu\text{g/mL}$ significantly reduced *Osx* expression at 30 h (Figure 5d) and *Alp* expression at day 7 (Figure 5f), indicating nonspecific gene suppression by the HBP core treatment. Next, an assessment of mineralized nodule formation in osteoblast cultures was conducted by the Alizarin Red S staining. Cells were cultured with osteogenic differentiation medium for 14 days. Figure 5g shows that the delivery of polyOEGMA-HBP and polyDMSO-HBP alone had no impact on osteoblast mineralization after 14 days in culture, while HBP core both at 2.5 and 10 $\mu\text{g/mL}$ resulted in reduced alizarin red positive colonies, indicating a decrease in mineralization (Figure 5g,h). Although we have demonstrated that HBP core at low concentration does not show toxicity for cell survival (Figure S2c), the long-term treatment with low doses of the HBP core shows negative impact on osteogenic ability of the primary osteoblasts. These results suggest that biocompatible shells such as polyOMEGA and polyDMSO are crucial to minimizing undesired cytotoxicity and nonspecific gene suppression of the cationic HBP core.

Polymer-Mediated RNAi Attack on *Runx2* Expression.

The efficacy of cationic polymer-based RNAi against *Runx2* expression in osteoblasts was determined by using quantitative real-time PCR. Cells were treated with the siRNA polyplexes and incubated in media containing 10% serum for 24 h prior to rhBMP-2 delivery. Analysis of *Runx2* gene expressions was conducted after 6 h of the rhBMP-2 treatment (Figure 6a). The commercially available lipid-based siRNA delivery reagent, *Lipofectamine*, was used as a reference for gene silencing. For transfection, the siRNA-*Lipofectamine* complexes were incubated in serum-free media for 6 h. The delivery of scramble siRNAs by cationic polymers was conducted to determine if the gene silencing capabilities of polymer-based RNAi were sequence specific. Results indicated that scramble siRNAs delivered by polyOEGMA-HBP and polyDMSO-HBP, with a polymer/siRNA weight ratio at 20/1, had no significant effect on *Runx2* expression compared to cells receiving rhBMP-2 treatments without siRNA (Figure 6b). The cells treated with the various siRNA polyplexes showed a significant decrease in *Runx2* expression compared to rhBMP-2 treated cells without siRNA (Figure 6c). For polyOEGMA-HBP, a polymer/siRNA weight ratio at 5/1 resulted in a significant reduction in *Runx2* expression of $59.2 \pm 13.8\%$ ($p < 0.05$), while a polymer/siRNA weight ratio at 20/1 resulted in *Runx2* knockdown of $18.9 \pm 27.5\%$ ($p = 0.38$). For polyDMSO-HBP, polymer/siRNA weight ratios both at 5/1 to 20/1 exhibited significant *Runx2* mRNA reductions of $77.2 \pm 2.9\%$ ($p < 0.01$) and $55.1 \pm 8.7\%$ (p

< 0.05), respectively. Each polymer showed silencing efficiencies comparable to *Lipofectamine* ($68.2 \pm 11.9\%$ gene suppression). It is notable that *Runx2* mRNA levels were knocked down by polymer-based RNAi treatments to levels consistent with untreated cells. For HBP core, polymer/siRNA weight ratios ranging from 5/1 to 20/1 elicited significant reductions in *Runx2* expression of $53.6 \pm 15.7\%$ ($p < 0.05$) and $72.3 \pm 15.3\%$ ($p < 0.01$), respectively (Figure S7). This is likely due to the toxic effects caused by HBP core based on the results shown in Figure 5 and thus nonspecific reductions in gene expression. Additionally, *Runx2* siRNA delivery with core-shell structured polymers had no significant effect on *Osx* expression at 6 h after the treatment (Figure S8), indicating that RNAi using polymers developed here allows targeted gene-specific silencing.

EFFECT OF POLYMER-MEDIATED *RUNX2* KNOCKDOWN ON OSTEOBLAST DIFFERENTIATION

To address whether the polymer-based RNAi can alter biological function of osteoblasts, osteogenesis was induced with rhBMP-2 and cultures were treated with siRNA polyplexes against *Runx2* every 48 h cycle for 14 days, then deposition of mineral was assessed by Alizarin red S staining (Figure 7a). Results indicated that scramble siRNAs delivered by polyOEGMA-HBP and polyDMSO-HBP, at a polymer/siRNA weight ratio at 20/1, or treatment of siRNA alone without polymers (Figure S9) had no significant effect on mineral deposition compared to rhBMP-2 treated cells without siRNA. RNAi treatments against *Runx2* with polyOEGMA-HBP and polyDMSO-HBP, with polymer/siRNA weight ratios at 20/1, resulted in significant reductions in mineral deposition in osteoblasts compared to cells receiving rhBMP-2 treatments without siRNA (Figure 7b,c). Areas of nodules in the treatment groups were similar to those in rhBMP-2 untreated cells, and this level of reduction was comparable with that of *Lipofectamine* (all $p < 0.01$). These results suggest that repeating the polymer-based siRNA transfection several times on the same cells provides long-term silencing efficiency. Overall, the gene silencing efficacy of each core-shell structures was comparable to *Lipofectamine*. Further, there were no apparent cytotoxic effects by both polyOEGMA-HBP and polyDMSO-HBP treatment. Therefore, the hyper-branched core-shell structures may have potential for efficient gene silencing without any undesirable cytotoxic effects.

CONCLUSIONS

In conclusion, cationic HBP were synthesized by SCVP of an ATRP inimer containing quaternary ammonium moiety with a cationic charge. By tuning the ratio of activator to deactivator, the DB of the HBP could be tuned from 16% to 34%. The cationic HBP showed efficient siRNA complexation but high cytotoxicity. In order to improve biocompatibility, two types of core-shell structures polyOEGMA-HBP and polyDMSO-HBP were synthesized from the cationic HBP core with DB of 34%. Both type of core-shell structures displayed improved biocompatibility up to 1 mg/mL with SH-SY5Y epithelial neuroblastoma cells. Due to the lower steric hindrance of polyDMSO compared to polyOEGMA shell, the polyDMSO-HBP polymer showed much higher siRNA complexing efficiency than polyOEGMA-HBP. The efficacy of cationic polymer-based RNAi against

Runx2 expression in osteoblasts was determined by using quantitative real-time PCR and the osteoblast differentiation was determined using Alizarin red S staining. The analysis indicated that the HBP core showed nonspecific gene suppression while both the core-shell structures showed long-term specific gene suppression against *Runx2* expression with efficacy comparable to that of commercial agent *Lipofectamine*. This work is the first example of successful use of the new biocompatible polymer polyDMSO for gene knockdown and the use of HBP based core-shell structures showed promise for long-term treatment of HO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
SEC traces of the cationic hyperbranched polymers with different DB.

Figure 2.

(a) SEC traces of polyOEGMA–HBP star polymers and (b) SEC traces of polyDMSO–HBP star polymers.

Figure 3. Cytotoxicity of SH-SY5Y epithelial neuroblastoma cells treated with (a) polyOEGMA–HBP with shell DP 5 and (b) polyDMSO–HBP with shell DP 5. HBP core with DB of 34% were used for grafting shell.

Figure 4.

Agarose gel of (a) core-shell structure polyOEGMA-HBP and (b) core-shell structure polyDMSO-HBP at weight ratios of polymer/siRNA from 0.16/1 to 400/1. Both polymers with shell DP = 5.

Figure 5.

Effects of HBP polymers and core-shell structures on osteoblast proliferation and differentiation at 2.5 $\mu\text{g/mL}$ (a,c,e,g) and 10 $\mu\text{g/mL}$ (b,d,f,h). (a,b) The rate of cell growth by polymer treatments after 5 days in culture. (c,d) Effect of polymers on both *Runx2* and *Osx* gene expressions compared to untreated groups at 30 h. (e,f) The gene expression levels after polymer treatment at day seven. (g,h) The effect of treatment of polymers on osteoblast mineralization after 14 d in culture in alizarin red positive colonies. Data expressed as mean $\pm$ SD of three replicate determinations. Significant differences between PBS-treated cells vs polymer-treated cells. ** $p < 0.01$, * $p < 0.05$.

Figure 6.

Cationic hyperbranched polymer-based RNAi against *Runx2* in primary osteoblasts by delivery of *Runx2* siRNAs at 20 pM doses. (a) Schematic of experimental time course. The siRNA polyplexes were delivered 24 h prior to delivery of recombinant human bone morphogenetic protein 2 (rhBMP-2, 100 ng/mL). Analysis of mRNA expression was conducted after 6 h of treatment by rhBMP-2. (b) Gene silencing effects on *Runx2* mRNA expressions by polymer/scramble siRNAs polyplexes compared to rhBMP-2 treated cells without siRNA. (c) Gene silencing effects on *Runx2* mRNA expressions by polymer/siRNAs polyplexes. Data expressed as mean $\pm$ SD of three replicate determinations. ** $p < 0.01$, * $p < 0.05$, versus rhBMP-2 treated cells without siRNA.

Figure 7.

Alizarin red staining was performed after siRNA against *Runx2* transfections. (a) Schematic of experimental time course. RNAi treatments against *Runx2* were delivered 24 h prior to delivery of rhBMP-2. Cell culture media were refreshed in conjunction with RNAi treatments and rhBMP-2 every 2 days for the duration of the study. After 14 d in culture, mineral deposition in osteoblasts was assessed by Alizarin red staining. (b) The scramble siRNAs delivered by polyOEGMA-HBP and polyDMSO-HBP, a polymer, siRNA weight ratio at 20/1, had no significant effect on mineral deposition compared to rhBMP-2 treated cells without siRNA. RNAi treatments against *Runx2* with polyOEGMA-HBP and polyDMSO-HBP, a polymer/siRNA weight ratio at 20/1, resulted in significant reductions in mineral deposition in osteoblasts compared to cells receiving rhBMP-2 treatments without siRNA. (c) The percentage of nodule area was measured using ImageJ. Data expressed as mean $\pm$ SD of three replicate determinations. ** $p < 0.01$, versus rhBMP-2 treated cells without siRNA.

Scheme 1. Synthesis of Cationic Hyperbranched Polymers^a

^a(a) Synthesis of HBP. Conditions: [inimer]/[ascorbic acid]/[CuBr₂]/[TPMA] = 50/*x*/4/6 (35 °C, DMSO, 38 wt %, *x* = 0.3, 1.3, or 4.4). (b) Mechanism of AGET ATRP involving activation and deactivation. (c) Reaction mechanism of the first three steps in a SCVP process.

Scheme 2. Schematic Representation of Two Types of Core-Shell Systems, polyOEGMA-HBP and PolyDMSO-HBP

Table 1.

Molecular Weight, Dispersity (), and Degree of Branching (DB) of Cationic HBPs

reaction #	[Asc. A.]/[CuBr ₂]	conversion	M_n^b	b	DB
HBP-1	0.3/4	98.3%	5,700	1.78	34%
HBP-2	1.3/4	98.1%	19,000	1.81	22%
HBP-3	4.4/4	97.0%	14,200	1.49	16%

^aFeed ratio of [ascorbic acid] to [CuBr₂]^bNumber-averaged molecular weight and MW dispersity determined by SEC-MALS in pure DMF

Table 2.**Two Types of Core–Shell Structures^a**

#	shell type	shell DP ^b	core–shell M_{nSEC}	core–shell	core–shell M_{nLS}	zeta ^c (mV)	size ^d (nm)
A	polyOEGMA	5	8500	1.37	31500	+10.1	10.6
B	polyOEGMA	48	69300	1.21	95100	+1.3	14.0
C	polyOEGMA	95	127000	1.33	1500000	+1.2	20.1
D	polyDMSO	5	2800	1.41	15900	+12.7	7.7
E	polyDMSO	35	20800	1.73	41300	+1.49	10.5
F	polyDMSO	45	28600	1.73	62100	+1.38	12.6

^a All structures were based on core with DB of 34%.^b Degree of polymerization of each side chains.^c Surface charge of the core–shell structures.^d Volume-mean size determined by DLS.