Supplementary information

Cationic Hyperbranched Polymers with Biocompatible Shells for siRNA Delivery

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1. Calculation of Degree of Branching (DB)

The calculation was based on previous reports.[1-3] Figure S1 shows the ^{1}H NMR of a hyperbranched polymer (HBP) with DB of 34 % purified with dialysis.

The HBP structure is composed of five different moieties (Scheme S1):

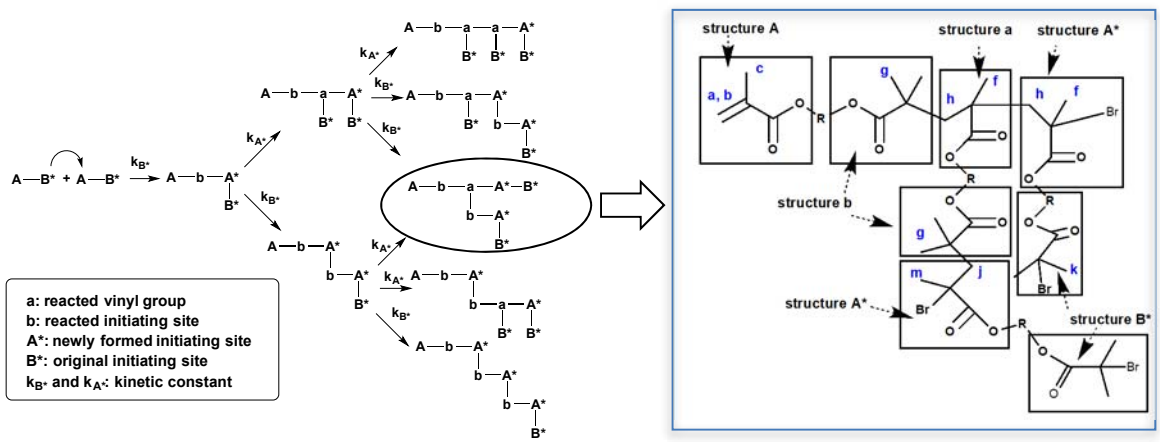
Structure A: unreacted vinyl group

Structure a: reacted vinyl group

Structure b: reacted initiating site

Structure A*: newly formed initiating site

Structure B*: original initiating site



Scheme S1. Illustration of all moieties of the HBP with rate constants, k_{A*} and k_{B*} in the initial steps in the polymerization. ($r=k_{A*}/k_{B*}$)

Relative amounts of each moiety in the HBP structure was calculated by integration of NMR peaks shown in Figure S1:

Area(**j**) = $2N_{A*}$;

Area(**c**+**f**+**g**+**h**+**k**+**m**)= $3N_a + 6N_b + 3N_{A*} + 6N_{B*} + 2N_a + 3N_A$;

Area(**a**+**b**)= $2N_A$;

$N_{A*} = N_b$;

$N_{B*} = N_a + N_A$;

With the above six equations, N_{B*} and N_B can be calculated. Since conversion of

vinyl groups approached essentially 100 %, Conv. A was assumed as 1, to simplify the calculation.[1, 2] With the calculated results, DB can be calculated according to the following equations:

$$f_{B*} = \frac{N_{B*}}{N_{B*} + N_B}$$

$$r = \frac{Conv.A + f_{B*} - 1}{-lnf_{B*} + f_{B*} - 1} = \frac{e^{-z}}{e^{-z} + z - 1}$$

$$DB = 2e^{-z}(1 - e^{-z})$$

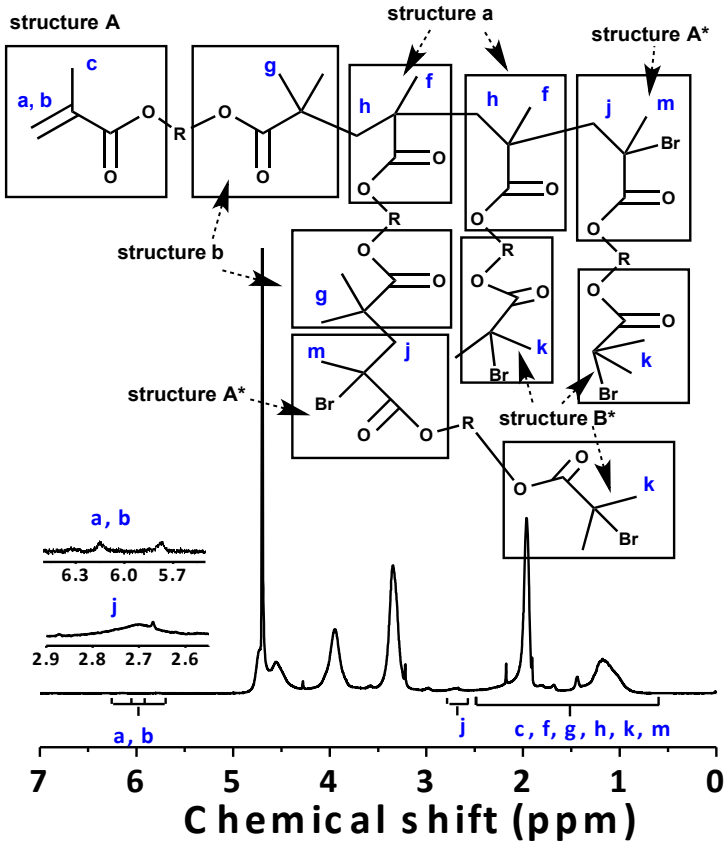


Figure S1. ^{1}H NMR spectrum of HBP (DB=34 %) with assigned NMR peaks.

The relative NMR integrations of Area(**j**), Area(**c**+**f**+**g**+**h**+**k**+**m**) and Area(**a**+**b**) for HBP with different DB are listed below in Table S1.

Table S1. Relative NMR integration areas and calculated DB.

Polymers	Area(**j**)	Area(**c**+**f**+**g**+**h**+**k**+**m**)	Area(**a**+**b**)	DB
HBP-1	1	27.0	0.026	34 %
HBP-2	1	43.4	0.151	22 %
HBP-3	1	67.1	0.099	16 %

2. Characterization of cationic hyperbranched polymers (HBP).

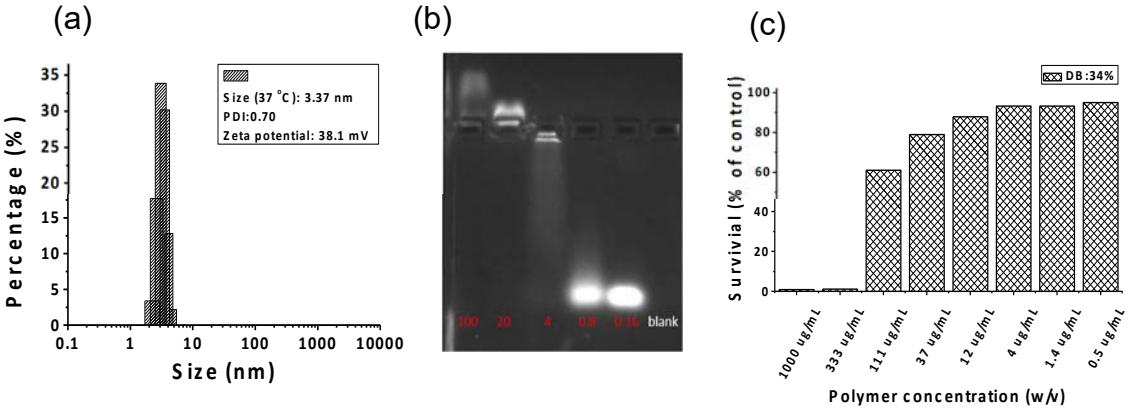


Figure S2. (a) Volume-mean size distribution by DLS of cationic HBP with DB 34%. (b) Agarose gel analysis of cationic HBP/siRNA complexes (weight ratio of polymer/siRNA from left to right: 100/1, 20/1, 4/1, 0.5/1, 0.16/1 and blank) (c) Cytotoxicity data of cationic HBP with SH-SY5Y cell. The polymer became toxic at concentration over 333 μg/mL, indicating that a biocompatible corona is needed to reduce the toxicity of the naked cationic core.

3. Characterization of core-shell structures.

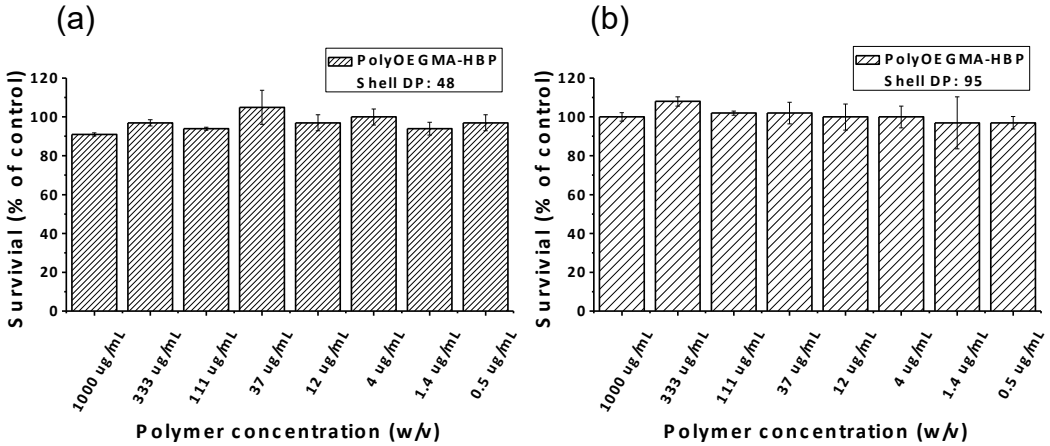


Figure S3. Cytotoxicity of the polyOEGMA-HBP polymers with different shell DPs grafted from a cationic core with DB of 34 %. (cells tested: SH-SY5Y) (a) Shell DP=48, (b) shell DP=95.

Core-shell structure polyOEGMA-HBP with thicker corona (shell DP = 48 or 95)

also showed low cytotoxicity towards cell SH-SY5Y with concentration up to 1 mg/mL.

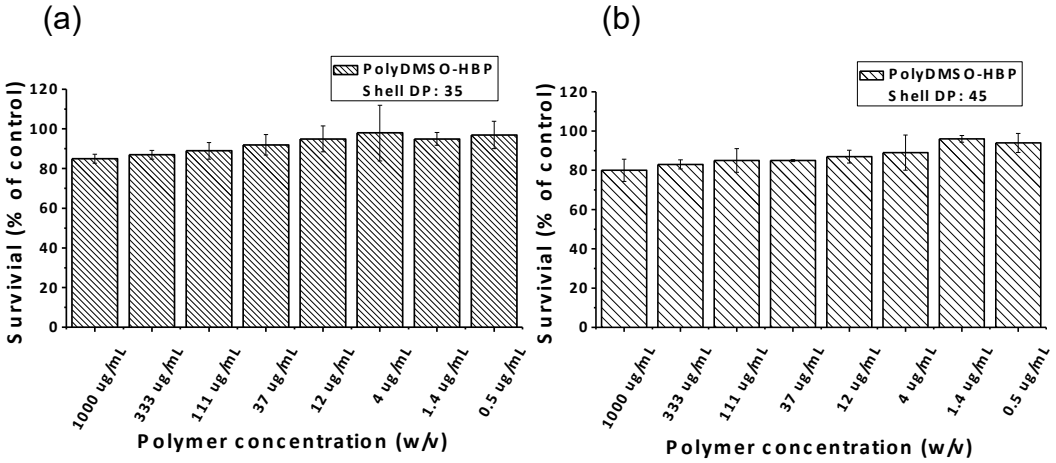


Figure S4. Cytotoxicity of the polyDMSO-HBP polymers with different shell DPs grafted from a cationic core with DB of 34 %. (cells tested: SH-SY5Y) (a) Shell DP=35, (b) shell DP=45.

Similarly, core-shell structure polyDMSO-HBP with thicker corona (shell DP = 35 or 45) also showed low cytotoxicity towards cell SH-SY5Y with concentration up to 1 mg/mL. These results suggest that polyDMSO provides biocompatible systems.

The siRNA complexing ability of polyOEGMA-HBP and polyDMSO-HBP with thicker corona was examined using agarose gel (Figure S5). PolyOEGMA-HBP with a shell DP of 48 did not show complexation with siRNA at weight ratio of polymer/siRNA lower than 200/1 and no complexes was formed even at a weight ratio of 400/1 for polyOEGMA-HBP with shell DP of 95. In comparison, polyDMSO-HBP with shell DP of 45 started to form complexes at a weight ratio of 40/1 (5 times lower than polyOEGMA-HBP with similar shell DP). This results indicated that due to the lower steric hindrance of polyDMSO, core-shell structures polyDMSO-HBP was more efficient than polyOEGMA-HBP for complexing siRNA.

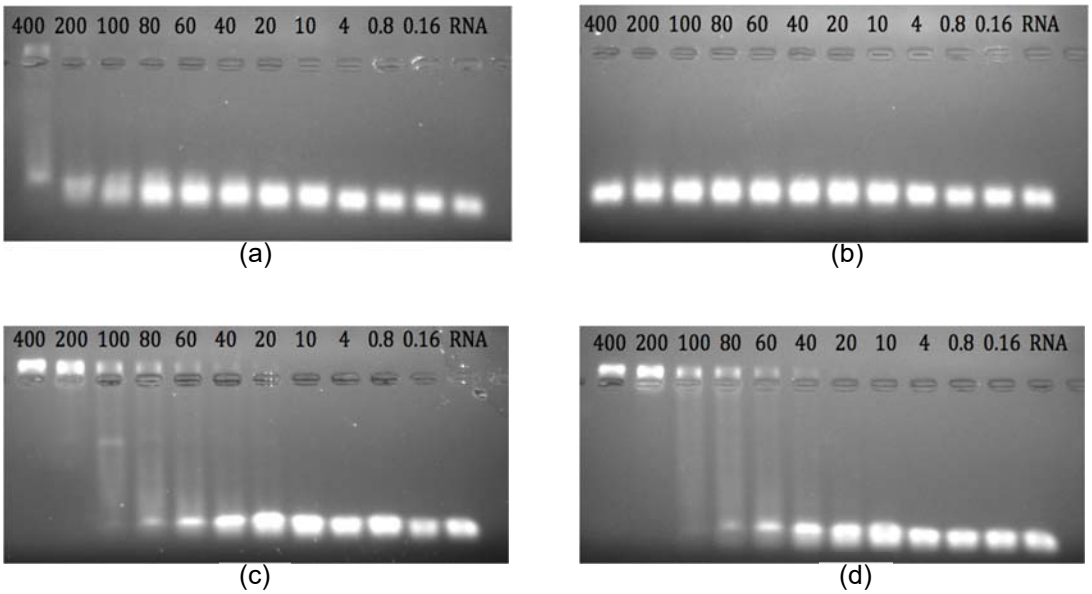


Figure S5. Agarose gel analysis of core-shell structure polyOEGMA-HBP with shell DP 48 (a) and 95(b); agarose gel of core-shell structure polyDMSO-HBP with shell DP 35 (c) and 48(d). Weight ratios of polymer/siRNA range from 0.16/1 to 400/1.

4. Stability of siRNA complexes

The stability of siRNA polyplexes was assessed by agarose gel electrophoresis. The result showed that the incubation of naked siRNA with culture media containing 10% FBS resulted in clear bands for 4h, and after 12 h, blurred bands were observed up to 48 h (Figure S6a). For polyOEGMA-HBP, siRNA complexes were stuck in the wells and prevented the internalization of siRNA into agarose gel, but the bands became blurred after 12 h (Figure S6b). By contrast, polyDMSO-HBP/siRNA polyplexes showed slightly blurred bands after 12 h, but the formation of polyplexes remained detectable up to 48 h (Figure S6c). No free siRNA was observed both in polyOEGMA-HBP and polyDMSO-HBP polyplexes up to 48 h. These results suggest that polyDMSO-HBP polyplexes are more stable than polyOEGMA polyplexes in culture media containing 10% FBS.

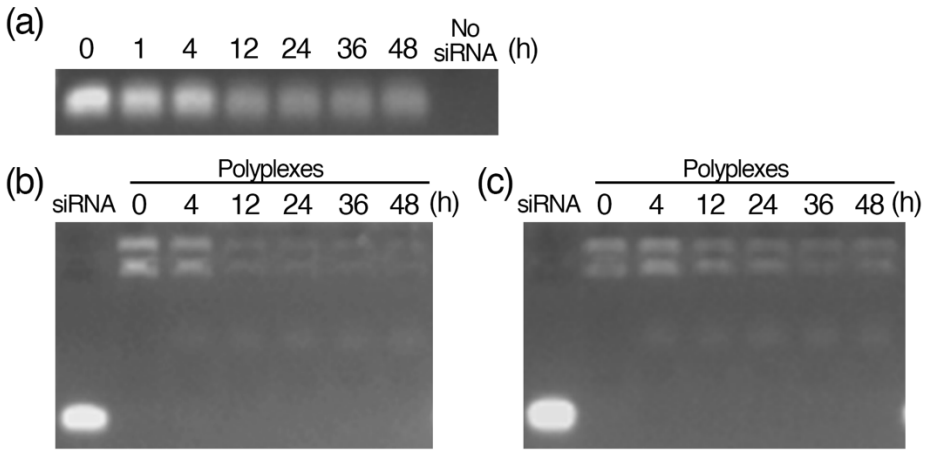


Figure S6. The stability of siRNA polyplexes in culture media containing 10% FBS up to 2 days. (a) Naked siRNA, (b) polyOEGMA-HBP and (c) polyDMSO-HBP at polymer/siRNA weight ratio of 400/1 were incubated for 4, 12, 24, 36 and 48 h at 37 °C. The stability of complexes was analyzed by electrophoresis on agarose gel stained with ethidium bromide. For better comparison, each gel was put in the same image.

5. siRNA delivery for gene knockdown

As shown in Figure S7, for HBP core, polymer/siRNA weight ratios ranging from 5/1 to 20/1 caused significant reductions in *Runx2* expression of 53.6 ± 15.7 % ($p < 0.05$) and 72.3 ± 15.3 % ($p < 0.01$), respectively. This is likely due to the toxic effects caused by HBP core, based on the results shown in Figure 5, and thus non-specific reductions in gene expression.

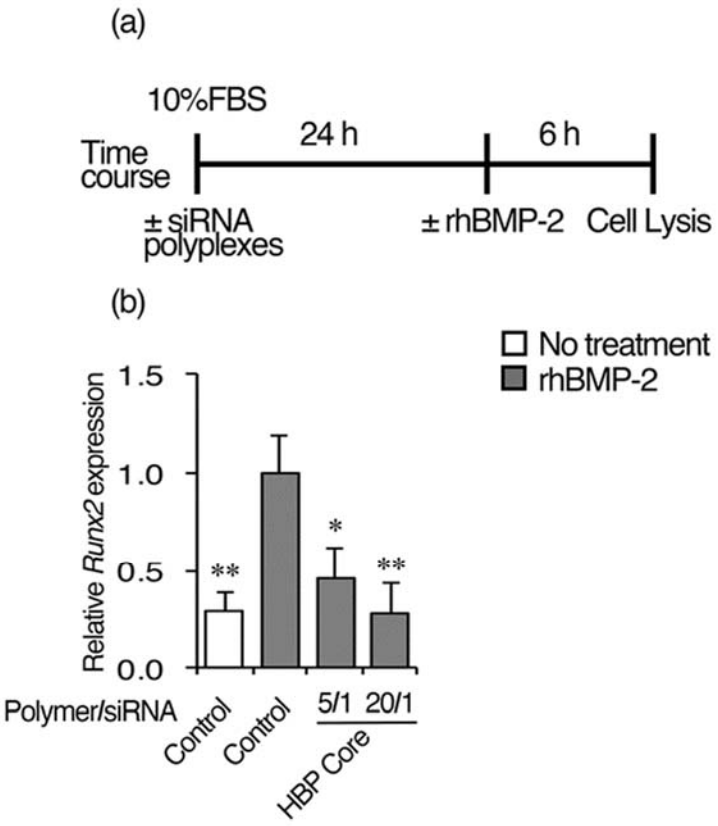


Figure S7. The HBP core-based RNAi against *Runx2* in primary osteoblasts by delivery of *Runx2* siRNAs at 20 pM doses. (a) Schematic of experimental time course. The siRNA polyplexes were delivered 24 h prior to delivery of recombinant human bone morphogenetic protein 2 (rhBMP-2, 100 ng/mL). Analysis of mRNA expression was conducted after 6 h of treatment by rhBMP-2. (b) The effects on *Runx2* mRNA expressions by HBP Core/siRNAs polyplexes. The polymer/siRNA weight ratios ranging from 5/1 to 20/1 elicited significant reductions in *Runx2* expression. Data expressed as mean ± SD of three replicate determinations. $^{**}p<0.01$, $^{*}p<0.05$, vs rhBMP-2 treated cells without siRNA.

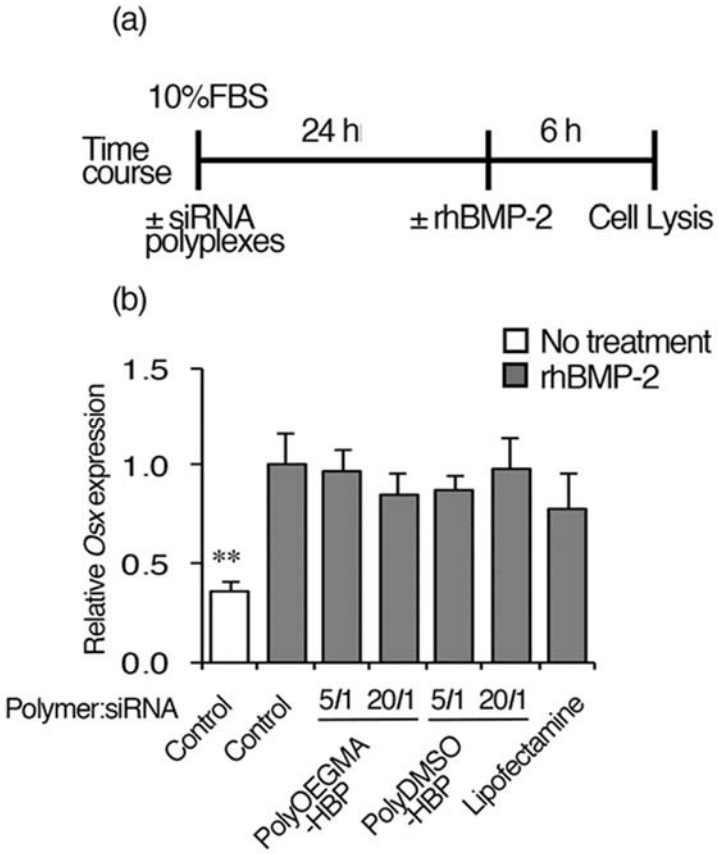


Figure S8. Delivery of *Runx2* siRNAs with core-shell structured polymers had no significant effect on *Osx* expression at 6 h after the treatment. (a) Schematic of experimental time course. The siRNA polyplexes were delivered 24 h prior to delivery of rhBMP-2. (b) Gene silencing effects on *Osx* mRNA expressions as a consequence of RNAi targeting *Runx2*. This result indicated that RNAi using core-shell structured polymers allows targeted gene-specific silencing. Data expressed as mean ± SD of three replicate determinations. $^{**}p<0.01$, vs rhBMP-2 treated cells without siRNA.

The *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.

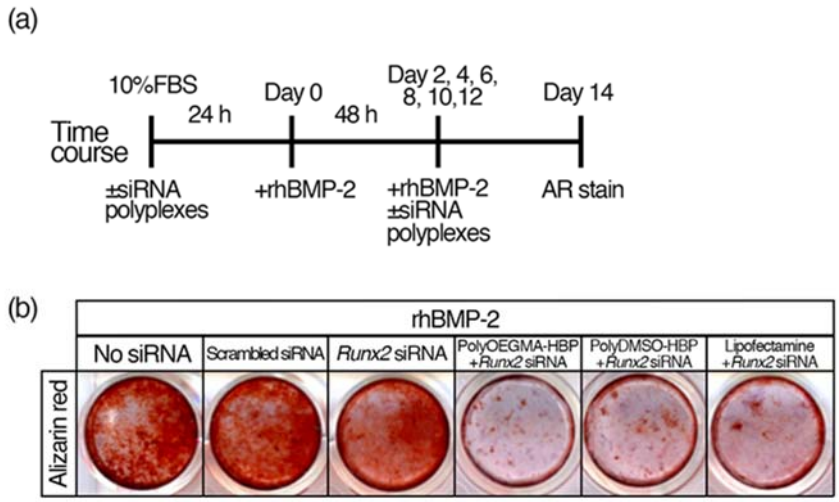


Figure S9. Alizarin red staining was performed after siRNA against *Runx2* transfections. (a) Schematic of experimental time course. RNAi treatments against *Runx2* were delivered 24 h prior to delivery of rhBMP-2. Cell culture media were refreshed in conjunction with RNAi treatments and rhBMP-2 every 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 cells receiving rhBMP-2 treatments with *Runx2* siRNA alone had no significant effect on mineral deposition compared to rhBMP-2 treated cells without siRNAs. RNAi treatments against *Runx2* with PolyOEGMA-HBP and PolyDMSO-HBP, a polymer/siRNA weight ratio at 5/1, resulted in significant reductions in mineral deposition in osteoblasts compared to cells receiving rhBMP-2 treatments without siRNA.

As shown above, cells treated with *Runx2* siRNA alone had no significant effect on mineral deposition compared to cell not treated with siRNAs, indicating the knockdown process requires the co-delivery of cationic polymers.

5. References

（1） Yan, D.; Müller, A. H. E.; Matyjaszewski, K. Molecular Parameters of Hyperbranched Polymers Made by Self-Condensing Vinyl Polymerization. 2. Degree of Branching. *Macromolecules* **1997**, *30*, 7024-7033.

（2） Min, K.; Gao, H. New Method to Access Hyperbranched Polymers with Uniform Structure Via One-Pot Polymerization of Inimer in Microemulsion. *J. Am. Chem. Soc.* **2012**, *134*, 15680-15683.

（3） Graff, R. W.; Wang, X.; Gao, H. Exploring Self-Condensing Vinyl Polymerization of Inimers in Microemulsion to Regulate the Structures of Hyperbranched Polymers. *Macromolecules* **2015**, *48*, 2118-2126.