Selective local delivery of RANK siRNA to bone phagocytes using bone augmentation biomaterials

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Abstract

Fracture healing and fracture fixation in the context of osteoporosis is extremely difficult. To inhibit osteoclast-induced bone resorption and associated implant loosening in this pathology, we describe a local delivery strategy to delivery RNA interfering technology to bone sites to target and down-regulate osteoclast formation and function. Resorbable polymer, poly(lactic-co-glycolic acid) (PLGA) microparticles were exploited as a passive phagocyte-targeting carrier to deliver RANK siRNA to both osteoclast precursors and osteoclasts—the professional phagocytes in bone. These natural phagocytes internalize micron-sized particles while most other non-targeted cells in bone cannot. PLGA-siRNA microparticles were dispersed within biomedical grade calcium-based injectable bone cement clinically used in osteoporosis as a bone augmentation biomaterial for fragility fracture prevention and fixation. siRNA released from this formulation in vitro retains bioactivity against the cell target, RANK, in cultured osteoclast precursor cells, inhibiting their progression toward the osteoclastic phenotype. These data support the proof-of-concept to utilize a clinically relevant approach to locally deliver siRNA to phagocytes in bone and improve fragility fracture healing in the context of osteoporosis. This local delivery system delivers siRNA therapeutics directly to osteoporosis sites from clinically familiar injected bone augmentation materials but could be extended to other injectable biomaterials for local siRNA delivery.

Keywords: siRNA, PLGA microparticles, Calcium phosphate bone cement, Bone augmentation

1. Introduction

Osteoporosis is a chronic disease characterized by low bone density and quality, responsible for fragility fractures in the hip, wrist and spine, and severely impairing patients' quality of life [1]. Most osteoporotic fractures require operative reduction and stabilization, but fracture healing processes in the context of ongoing osteoporosis pathology are problematic. Low bone quality and excessive bone resorption decrease the mechanical strength necessary for acceptable, durable fracture healing. Clinical approaches to improve bone quality in osteoporosis fixation include reinforcement of porous, weakened bones prior to fracture or device implantation (i.e., prophylaxis) by direct augmentation of bone around the fracture and fixation hardware using injected prosthetic bulking materials [2–6]. The technique utilizes various biomaterials, including polymethylmethacrylate (PMMA), calcium phosphate substitutes (as bone cements, shaped blocks, coatings), various bone grafts, and modified implants [7]. Calcium phosphate cement (CPC), a ceramic chemically similar to natural bone's mineral component, has attracted attention due to several unique properties, including intrinsic resorption and replacement with nature bone, deployment as viscous setting fluid matrices by local injection and acceptable in situ biocompatibility [8–10]. In vivo degradation of CPC-based cements can vary widely depending on formulation and chemistry, induced by both passive physiologic dissolution and active cell-mediated phagocytosis [11]. To date, substantial experience supports the feasibility and value of using CPC as a drug carrier in bone augmentation. Pharmacologically active molecules of diverse chemistries are readily distributed within the liquid bone cement formulation prior to setting. Several drugs have been formulated for release from CPC, including antibiotics to decrease post-surgical infections [12,13], anticancer drugs to reduce tumorogenesis [14] and growth factors to promote bone healing [15]. However, release rates of encapsulated drugs are limited since most CPC formulations are dense monoliths and...

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in vivo release is slow for degradation-controlled release. To enhance release and CPC degradation, soluble porogens are incorporated into the CPC to more rapidly dissolve and enhance CPC porosity and dissolution, and drug release [8].

Degradable poly(lactic-co-glycolic acid) (PLGA) particles have been incorporated into cement to deliberately increase porosity and interconnectivity upon their degradation, facilitating further active and passive in vivo degradation of cement and enhancing bone in-growth at surgical sites [8,16]. Resultant changes of CPC handling properties and cement setting are maintained within desired workable ranges [8]. This provides an opportunity to load both the CPC matrix and encapsulated PLGA particles with drug loads for controlled release strategies from the injectable hybrid biomaterial. PLGA is a well-studied degradable polymer for particle-based formulations: FDA-approved PLGA microsphere drug delivery products include Lupron Depot® (Abbot Laboratories), Trelistra® Depot (Watson Pharmaceuticals), Risperdal® Consta™ (Ortho-McNeil-Janssen Pharmaceuticals) and Bydureon™ (Amylin Pharmaceuticals). Bioactive molecule microencapsulation for use in both animals and humans and versatile capacity for sustaining long-term controlled drug release in these therapeutic systems is well-established [17,18]. As a dual-use drug delivery mechanism, PLGA particles have also been tested in combination with CPC to deliver bone growth factors (rhBMP-2) while also acting as a porogen [19].

After a typical device implantation, large numbers of differentiating osteoclast precursors, monocyte macrophages, are recruited to the peri-implant area, especially at the bone—implant interface [20,21]. In osteoporosis and osteopenias, enhanced bone resorption and osteoclastogenic activities often correlates with reduced bone—implant interface area and results in aseptic loosening and finally prosthetic failure. Specifically suppressing osteoclastic bone resorption activity in peri-implant area could improve initial implant stabilization. Significantly, smaller PLGA microparticles (i.e., <10 μm diameter) are selectively scavenged by phagocytic cells, providing a particle size—selective cell—specific drug targeting and dosing mechanism [22]. Therefore, specific osteoclast and phagocyte cell uptake can be achieved by controlling PLGA particle sizes to those internalized by professional phagocytes while avoiding other intrinsically low-phagocytically active cell types (i.e., fibroblasts, osteoblasts, progenitor cells) in the tissue bed.

Gene silencing using siRNA has many potential therapeutic applications due to several advantages intrinsic to RNA interference (RNAi) [23]. siRNAs are well-known for their very short circulating half-lives in vivo [24], and current challenges in systemic targeting from parenteral siRNA formulations [25]. These unresolved issues have produced the current emphasis on local delivery of therapeutically active siRNA as a more direct clinical goal [26], also in many possible bone targets [27]. Two cytokines—RANK ligand (RANKL) and macrophage colony-stimulating factor (M-CSF)—are essential for osteoclastogenesis [28,29]. RANKL interactions with RANK receptor on the surface of osteoclast precursors are critical for expression of osteoclast—specific genes [30], bone resorption and survival of mature osteoclasts [31]. Osteoclastogenesis is negatively regulated by osteoprotegerin (OPG), which is also expressed by stromal cells and osteoblasts [32]. OPG is a decoy receptor that competes with RANK for binding RANKL [33]. In this RANKL/RANK/OPG axis, positive regulator RANKL and negative regulator OPG are coordinated through RANK interaction on osteoclast precursor cells in bone sites to regulate normal bone formation and degradation. We have shown recently that siRNA against RANK provides potent in vitro inhibition of osteoclast phenotypic functions and osteoclast-mediated bone resorption [34]. Here we describe a strategy to deliver RANK siRNA—loaded PLGA microspheres resident in an injectable CPC augmentation matrix for local delivery to low-density bone and specifically for suppressing bone—resident osteoclast formation and bone resorption activity in osteoporotic sites.

2. Materials and methods

2.1. Reagents

PLGA (50:50 monomer ratio, molecular weight (MW) 57 kDa) with lauryl ester end groups was purchased from Lakeshore Biomaterials (AL, USA). Branched poly-ethyleneimine (bPEI) (MW: 25 kDa) and poly(vinyl alcohol) (PVA, average MW 30–70 kDa) were purchased from Sigma—Aldrich (USA). RNase-free water was prepared using diethyl pyrocarbonate (DEPC) (Sigma—Aldrich), RANK siRNA (sense 5'-GGCGACACUUCCAUCAAUC AU-3', antisense 5'-UUAU GGAGUGAAGUCUGCGUU-3') and RNase-free siRNA buffer were purchased from Dharmacon (CO, USA). DNA substrate for the siRNA molecule against RANK (siRNA analog) (5'-GGCCA GACCTTCATCCATAT-3', 5'-TATGCAGAACGCTGCGC-3') were purchased from Integrated DNA Technologies (IA, USA).

2.2. Primary murine cell harvesting and differentiation

C57Bl6 male mice (6–8-week—old, Jackson Labs, ME, USA) were maintained in a specific pathogen—free facility at the University of Utah. All procedures were performed as approved by the Institutional Animal Care and Use Committee, University of Utah. Bone marrow cells (BMCs) were harvested from murine tibia and femurs of C57Bl6 male mice and differentiated into osteoclast precursors using previously described methods [34]. Briefly, BMCs were cultured in n−MEM (Gibco) containing 10% FBS and 1% penicillin-streptomycin (for all cell cultures as “complete media”) overnight at a density of 1 × 10⁶ cells/ml. Non—adherent cells were harvested the next day and immediately seeded into 24—well tissue culture plates in complete media with 30 ng/ml M-CSF (R&D Systems) at a density of 1 × 10⁵ cells per well. After 2 days of culture, attached cells were used as osteoclast precursors. To generate osteoclasts, precursor cells were incubated in 200 ng/ml RANKL and 30 ng/ml M-CSF (R&D Systems) in complete media (defined as “osteoclast—specific media”), refreshed every other day [44]. RANKL was expressed using a previously described detailed procedure [34].

2.3. Reverse transcriptase—polymerase chain reaction (RT—PCR)

Total RNA was isolated using an RNeasy Mini Kit (Qiagen). Up to 4 μg of RNA was used to make cDNA with the SuperScript III 1st strand RT kit for PCR (Invitrogen). PCR primers were designed for RANK (5'-AGATGTTGTCGTGCCAGTCTTCCAT-3', 5'-ACACACTTCTTGCTGACTGGAGGT-3') and cyclophilin B (housekeeping control, 5'-ACGGCTTCCCCAGATGAACTCTA-3', 5'-GCGAAGCAAAAGGTTRCTTCCACT-3') using Primerquest software and purchased from Integrated DNA Technologies (USA). PCR was performed with Taq DNA polymerase in the iCycler Thermal Cycler (Bio—Rad). PCR products were analyzed on ethidium bromide—stained TBE—based 2% agarose gels. The electrophoresis was run at 100 V for 30 min and the gel was visualized with Bio—Rad imaging system.

2.4. Preparation of siRNA/bPEI complexes and siRNA transfection in vitro

To prepare siRNA/bPEI complexes at various cation/anion (NP) charge ratios, 2 μl of 10 μM RANK siRNA aqueous solution was mixed with 2 μl of bPEI solutions of different concentrations that yield final mass ranging from 0.016 to 0.16 μg. The complex solutions were kept at room temperature for 20 min. Then, 4 μl of each complex solution was used for electrophoresis on ethidium bromide—stained TBE—based 2% agarose gels. The electrophoresis was run at 100 V for 20 min, followed by the visualization with UV light to assess the formation of siRNA—bPEI complexes. Cell transfections with siRNA/bPEI complexes at fixed NP ratios in complete media were performed subsequently. siRNA/bPEI complexes for each well were prepared by mixing 2.5 μl of 20 μM siRNA aqueous solution with 1.6, 1.2, 0.8 and 0 μl of NP (NP 20, 15, 10 and 0) of 1 mg/ml bPEI, respectively, in a total volume of 20 μl with RNase—free water. After incubation at room temperature for 20 min, complete media was added to achieve the final volume of 1 ml, yielding a final amount of siRNA in each well of 0.67 μg. The dose was determined based on published results [34,35]. siRNA transfections were always performed in complete media. After 24—h incubation at 37 °C under 5% CO₂, culture media was refreshed with 1 ml of complete media and transfected cells were further incubated. RNA was harvested 2 days later and RANK expression was analyzed by PCR. [34]

2.5. PLGA microparticle preparation

Microparticles of PLGA were prepared using a known double—emulsion solvent evaporation technique [36]. In brief, siRNA or siRNA analog/bPEI complexes were prepared by mixing equal volumes of 33.5 μg of siRNA/siRNA analog and 1 mg/ml of bPEI in RNase—free siRNA buffer. Complex—containing PLGA microspheres were prepared by dispersing the siRNA/bPEI aqueous solution into a solution of 50 mg
PLGA dissolved in 2 ml methylene chloride with intense probe sonication (Biologics, Inc. Ultrasonic 3000, 20 s with sonication with 10 s pause). Then, the water/oil dispersion was added into the 4% PVA solution while vortexing. The final w/o/w emulsion was mixed into 4 ml of 0.6% PVA solution and set for 3–4 h at room temperature while stirring. The solidified microspheres were washed using RNase-free water three times followed by lyophilization.

2.6. PLGA particle characterization

2.6.1. Particle size distribution, zeta potential and surface morphology

Particle size distribution and zeta potential (surface charge) of PLGA microparticles with siRNA was determined using a Malvern Instruments Zetasizer Nano ZS (Westborough, USA) in 10 μs NaCl at pH 7.4. To further confirm measured particle sizes and surface morphology, scanning electron microscopy (SEM) was performed. PLGA microspheres were attached to a metal stub using conductive tape and coated with gold in a sputter coater. SEM images of coated samples were captured at 5 kV (Hitachi S2460N).

2.6.2. siRNA loading and encapsulation efficiency

Following a previously described procedure [17], 3–5 mg PLGA particles were dissolved in DMSO. The siRNA was extracted by adding equal volumes of Tris–EDTA buffer (10 μs Tris–HCl containing 1 ms EDTA, pH 7.4). Aqueous layers were removed, and siRNA concentration was measured using the QuantIT™ Picogreen™ assay (Invitrogen, USA). Encapsulation efficiency was calculated as the percent of the PLGA-extracted siRNA versus the initial feed siRNA amount. The siRNA was then precipitated from the aqueous solution by adding 0.3 M sodium acetate and 70% (v/v) ethanol. After 30 min incubation at –20 °C, the solution was centrifuged at 12,000 rpm at 4 °C for 30 min. The pellet was dried and analyzed on a 2% agarose gel.

2.6.3. Evaluation of in vitro siRNA controlled release

siRNA analog-loaded PLGA particles (1 mg) were suspended in 1 ml RNase-free siRNA buffer (pH 7.4), and kept at 37 °C under constant gentle shaking for four weeks. To collect the supernatant, the suspension was centrifuged at 12,000 rpm for 15 min, and supernatant was removed for analysis followed by replacement with the same volume of fresh buffer. The siRNA content in the supernatant was measured using the QuantIT™ Picogreen™ assay.

2.6.4. Cytotoxicity assay

Murine BMCs were cultured in osteoclast-specific media with 1 mg/ml PLGA particles loaded with RANK siRNA/bPEI compared with cells without treatment. After 3 day-incubation in 37 °C, cytotoxicity was measured using CellTiter-Blue Cell Viability Assay kit (Promega, USA).

2.6.5. Particle in vitro cell-specific uptake and intracellular degradation studies

Murine BMCs were cultured in complete media at 1 × 106 cells/ml for 24 h and then incubated with 1 mg/ml PLGA particles for 3 days in this media. After three washes with PBS, live cell images were subjected to microscopic observation. Fluorescent dye-labeled PLGA particles were prepared by adding 0.5% (weight percentage vs. PLGA) 6-coumarin dye to the methylene chloride phase used in the PLGA microparticle fabrication, and microparticles were fabricated as described (vida supra) [37]. Osteoclast precursor cells were seeded at 1 × 105 cells/ml and incubated with 1 mg/ml fluorescein labeled PLGA particles in a 24-well plate in complete media containing M-CSF and RANKL at 37 °C. Particle intracellular degradation studies were performed using fluorescence microscopy. Culture media was refreshed every other day. Transition of punctate into diffuse fluorescence indicates particle degradation within intracellular bodies.

2.6.6. Particle release of RANK siRNA and RANK knockdown efficiency in cell culture

Murine BMCs were incubated with PLGA particles in 24-well plates in osteoclast-specific media. The dose of siRNA per well (125 nm) was determined based on previous published results [34]. RANK siRNA-loaded PLGA particle-treated groups were compared with control groups treated with blank PLGA particles without siRNA, and also with siRNA analog-loaded PLGA particle-treated groups. PLGA particles were diluted in 1 ml osteoclast-specific media and added to osteoclast cell cultures. Total RNA was isolated 4 days post-transfection using an RNaseasy Mini Kit (Qagen). PCR products were analyzed on ethidium bromide-stained TBE-based 2% agarose gels and visualized under UV light.

2.7. Loading of siRNA-containing PLGA particles into CPC

A commercially available clinical grade injectable CPC, a calcium sulfate hardening paste (Bone Plast, BP070) was gift from BioMet Osteobiologics (NJ, USA). The product is moldable to any shape and can be resorbed in 6–8 weeks by osteoclastic activity and chemical dissolution. Pure CPC cement was prepared according to the manufacturer’s instructions and used as the control. PLGA micro particles were loaded into dry CPC powder at 10% (w/w). This mixture was added into sterile 5-ml syringes (Becton Dickinson, NJ) filled with aqueous solution provided in the kit to achieve the liquid/powder ratio of 0.35 ml/g [8]. Subsequently, thorough mixing required manual stirring within the syringe barrel for 60 s. The resulting CPC suspension containing PLGA microparticles was then injected into a clean rubber mold (circular cylinder shape: 6 mm diameter, 6 mm height) to cure CPC samples in this shape.

2.8. Characterization of the PLGA-modified CPC cement

2.8.1. Cement porosity

The total porosity of PLGA microparticle–loaded CPC was determined by heating CPC specimens at 650 °C for 2 h to pyrolyze the PLGA particles from the sample. CPC sample weight was measured before and after pyrolysis. Total porosity and macroporosity was calculated from the following equations [38]:

\[
e_{\text{macrot}} = \left( \frac{m_{\text{burnt}}}{m_{\text{pure CPC}}} \right) × 100% \quad (1)
\]

\[
e_{\text{macro}} = \left( \frac{m_{\text{burnt}}}{\rho_{\text{HAP}}} \right) × 100% \quad (2)
\]

Equation (1) calculates macropores produced by PLGA particle voids. Equation (2) yields the sample total porosity, including micropores, representing the porosity of the pure CPC sample. \(m_{\text{burnt}}\) is the weight of samples after PLGA removal by heating, \(v\) (cm³) is the volume of the sample, \(m_{\text{pure CPC}}\) is the weight of pure CPC sample, and \(\rho_{\text{HAP}}\) (g/cm³) is the density of hydroxyapatite (3.156 g/cm³) [39].

2.8.2. CPC mechanical testing

CPC samples were vacuum-dried overnight. Compression strength was measured using an Instron (Instron TT-CM; Instron, Canton, USA). Samples were placed in the mechanical testing bench and compressive strength was measured at a crosshead speed of 0.5 mm/min. Mechanical stiffness was determined and compared between CPC alone and 10:90 PLGA/CPC samples.

2.8.3. CPC composite matrix morphology

Cross-sections of CPC samples (vacuum-dried overnight) were visualized for porosity and morphology by SEM.

2.9. RANK knockdown efficacy by siRNA released from modified cement in vitro

In order to fabricate CPC composite wafers to deploy into 24-well plate cell cultures for siRNA release and bioactivity assays in cell culture, 1 mg of PLGA microparticles carrying 0.67 μg of siRNA were mixed thoroughly with 9 mg CPC powder (10:90) on a sterilized plastic weighing plate, and dissolved in aqueous solution to reach the same liquid/powder ratio of 0.35 ml/g as used for mechanical samples. A flat medicine spoon was used to mold the CPC mixture into a flat disc. After curing the CPC into a hard wafer, a razor blade was used to cut and remove pieces of the CPC wafer to fit into wells of 24-well culture plate. Murine bone marrow cells were then seeded directly onto these CPC wafers at 1 × 105 per well in the wells. Cells were then incubated with 30 ng/ml M-CSF in complete media for the first two days and followed by incubation with osteoclast-specific media. Cell RNA was harvested 6 days post-treatment and subject to PCR. PCR products were analyzed on ethidium bromide-stained TBE-based 2% agarose gels run at 100 V for 30 min and visualized with UV light.

2.10. Cell imaging

Fluorescent images of adherent cells in culture were captured using a Coolscope color CCD camera attached to a Nikon E600 microscope using Image Pro Plus 4.0 software. All images were captured using the same exposure time period and corrected for unequal field illumination.

2.11. Statistical analysis

Analysis of variance (ANOVA) followed by two-tailed student’s t-test was used for statistical analyses. All experiments were repeated three times with multiple replicates within each run. Results were considered statistically significant if \(p < 0.05\).

3. Results

3.1. Optimization of RANK siRNA/bPEI complexes

To determine the NP threshold for stable siRNA polyplex formation, different amounts of bPEI were mixed with 0.14 nmol RANK siRNA at NP ratios of 0, 0.5, 1, 2, and 5. Fig. 1A shows the migration of siRNA/bPEI complexes by gel electrophoresis. With NP ratios of 0, 0.5 and 1, siRNA bands migrate on the gel, indicating uncomplexed, excess siRNA. When the NP ratio was 2, the free
siRNA band density was substantially weaker. When the NP ratio was 5, no siRNA migrates freely in the gel, indicating that all siRNA molecules are entrapped in bPEI complexes through electrostatic interactions. Knowing that an NP value of 5 was the threshold, RANK expression was assayed after cells were incubated with complexes with NP ratios of 10, 15 and 20 for 2 days. RANK gene expression started to decrease when the NP ratio was 15, and significant down-regulation was shown when the NP was 20 (Fig. 1B). Therefore, an NP ratio of 20 was used for the following delivery experiments.

### 3.2. Characterization of PLGA microspheres

PLGA SEM images (Fig. 2A) revealed that microspheres displayed the expected spherical morphology with an average size of 5.45 ± 0.88 μm as determined from light scattering particle size analysis. The zeta potential of PLGA microparticles was −21.07 ± 1.42 mV under these conditions. The siRNA encapsulation efficiency in these PLGA particles using the double-emulsion method is 80.75 ± 4.1% (n = 3).

### 3.3. RANK knockdown effects, cytotoxicity and intracellular degradation of PLGA microspheres

Cytotoxicity from PLGA microparticle exposure to cells in culture was determined: no significant cell toxicity differences were observed between controls without treatment and cells treated with particles (Fig. 2B). After three days of incubation with...
the BMC pool, PLGA particles were observed in the cytoplasm of phagocytic cells, primarily monocyte macrophages (Fig. 2C), very comparable to phagocytosis results for purified primary monocyte macrophages incubated with PLGA particles (Fig. 2D). Gel electrophoresis showed that the charge and molecular weight of RANK siRNA extracted from PLGA particles was similar to the control stock siRNA (Fig. 2E). RNA was harvested from murine-derived primary bone marrow cells incubated with microparticles for 4 days and RANK expression was analyzed. Cells incubated with RANK siRNA-loaded PLGA particles showed significant down-regulation of RANK expression, compared with controls without microparticle incubation and cells treated with microparticles loaded with siRNA analogs as controls (Fig. 2F). After 12 days in culture, the majority of microspheres disappeared and the enclosed fluorescence marker was distributed evenly throughout the cell volume, indicating release by particle degradation (Fig. 2G).

3.4. In vitro release of siRNA analogs of RANK siRNA from PLGA microparticles

Murine RANK siRNA analog-loaded (i.e., oligoDNA) PLGA microparticles were used to evaluate the in vitro release profile of siRNA from microparticles due to similar physical chemistry and structure, higher stability and lower cost of oligoDNA compared to siRNA. Little or no DNA release was observed from PLGA microparticles over 50 days in culture media alone (avg. ± SD, n = 3, error bars represent SD).

3.5. Characterization of PLGA microparticle/CPC composite matrix

Table 1 shows calculated results for microparticle porosity and macroporosity (avg. ± SE, n = 3). Pure CPC samples had a porosity of 55%, while the PLGA-loaded CPC samples (10% w/w particle load) displayed a higher porosity of 63%. The macroporous fraction introduced by PLGA particles was 35%.

3.6. CPC morphology and mechanical characteristics

Fig. 4A and B show SEM images of the cross-sections of pure CPC and CPC with PLGA particles, respectively, within the cured 10:90 PLGA/CPC matrix. PLGA microparticles were distributed homogeneously within the CPC matrix (Fig. 4B). CPC matrix stiffness is expressed as maximum compressive load (N) versus maximum compressive extension (mm). Control samples (CPC alone) showed a stiffness of 251.8 ± 27.14 N/mm (avg. ± SD). The 10% PLGA-incorporated CPC samples had an average value of 200.5 ± 9.57 N/mm (avg. ± SD). No significant difference in stiffness was observed between the CPC alone and the 10:90 CPC samples (Fig. 4C).

3.7. Down-regulation of RANK gene expression by RANK siRNA-loaded PLGA/CPC composite microparticles

Murine BMC RNA was harvested 8 days after these cells were seeded on CPC wafers. PCR results show significant suppression of RANK expression in cells incubated with RANK siRNA-loaded PLGA/ CPC compared with control without particle treatments and siRNA analog-loaded PLGA/CPC matrices (Fig. 4D).

4. Discussion

Increasing incidence of osteoporotic fragility fractures and their prolonged healing requirements post-fixation constitute major clinical challenges that require improved clinical solutions for better osteoporosis patient outcomes. Though bisphosphonate drugs show some efficacy against generalized osteoporosis, their severe suppression of bone turnover largely inhibits fracture healing [40,41]. Currently, no therapy has yet produced significant improvements in osteoporotic bone augmentation for improved fragility fracture healing. RNAi is a new promising drug class with unique mechanisms to selectively target pathological pathways. Yet, effective siRNA delivery to target tissues is difficult, mandating primarily local or topical routing to produce therapeutic efficacy to date. Importantly, while siRNA has been targeted to bone systemically [42], siRNA has not yet been studied to ameliorate osteoclast-mediated bone loss in the context of fragility fractures and fixation tooling. This opportunity was explored in this study with in vitro knockdown of RANK in murine BMCs by siRNA using PLGA particle-loaded injectable bone augmentation biomaterials (CPC) clinically applied in osteoporosis as delivery matrices.

As RANK is recognized to play an essential role in regulating osteoclastogenesis, it is a natural target for siRNA in the context of osteoporosis therapy [43]. Activation of RANK by its ligand, RANKL, is required for the formation and activation of osteoclasts [44,45]. Our previous RANK siRNA delivery methods [34] show specific siRNA silencing of RANK in both macrophage-like secondary cell line cultures (RAW264.7) and primary murine BMC cultures. Significantly, RANK siRNA also inhibited both osteoclast formation and bone resorption in vitro. These data collectively support proof-of-concept to deliver therapeutic RANK siRNA to osteoclasts locally to inhibit their metabolic activity in bone without necrosis [34]. Therefore, this current study further developed this concept for locally delivered siRNA targeting RANK from injectable bone cement, assessing in vitro siRNA delivery and RANK knockdown efficacy from PLGA particles within CPC bone augmentation materials to target osteoclasts and osteoclast precursors and suppress osteoclast-mediated excessive bone resorption.

To increase the stability and loading efficiency of siRNA in PLGA microparticles, RANK siRNA was complexed with bPEI at different NP ratios (Fig. 1) because of bPEI’s known utility as a non-viral nucleic acid delivery vector [46,47,48]. The siRNA/bPEI

| Microporous cement | 0.2522 ± 0.0045 | 1.770 ± 0.016 | 55.02 ± 0.42 | – |
| 10:90 PLGA/CPC | 0.2241 ± 0.0066 | 1.572 ± 0.023 | 63.68 ± 0.56 | 35.23 ± 0.99 |
migration assay demonstrates that siRNA forms stable complexes with bPEI when the NP ratio ≥ 5. Transfection of RANK siRNA in mouse BMCS in serum-based cultures with these polyplexes was monitored by PCR two days post-transfection. Compared to controls without treatment, RANK gene expression in BMCS cultures showed no significant gene expression reduction when the NP ratio was 10, but began to decrease when the NP ratio equaled 15. Significant RANK gene silencing was achieved when the NP ratio reached 20, indicating that siRNA specifically down-regulates RANK message RNA expression in BMCS. Therefore, an NP ratio of 20 was used for subsequent experiments. RANK knockdown effect was comparable to use of commercial cationic DharmaFECT 4 transfection reagent in our previous published results [34].

For maximum particle loading and protection of siRNA payloads from nucleases, 4% (w/v) PVA was used for PLGA microparticle fabrication [36,49]. Slight modifications to the emulsion fabrication process, including the time and power of sonication, facilitated microparticle size control to within the desired range of 1–10 μm needed to selectively target particle uptake exclusively by professional bone phagocytes [22]. PEI has been proven previously to increase oligoDNA encapsulation in PLGA particles at least 2-fold relative to that without PEI, especially for pre-formed PEI-oligoDNA polyplexes, and to increase cellular accumulation of nanoparticles [50]. For this study, the use of bPEI improves the siRNA-PLGA particle encapsulation efficiency to 81%, comparable with previous published encapsulation efficiency of pre-formed siRNA-PEI in PLGA particles [50]. Cytotoxicity from siRNA-loaded PLGA particles was analyzed by culturing BMCS with PLGA particles in serum-containing complete media at minimum effective doses determined previously [34]. No significant cytotoxicity was observed (Fig. 2). Electrophoresis results showed that the extracted siRNA from these particles maintained a similar charge, migration and molecular weight as the uncomplexed siRNA. Consistent with literature precedent [22], microparticles were readily phagocytosed by monocyte macrophages compared to non-phagocytic cell cultures (Fig. 2C), establishing this particle size-dependent local targeting mode at peri-implant sites known to attract bone phagocytes. Gradual intracellular particle degradation was observed using fluorescence imaging. Complete microparticle degradation was achieved in 12 days of incubation in cell cultures (Fig. 2).

In vitro release profiles of DNA from PLGA microparticles have been shown to be dependent on the molecular mass of the PLGA; increasing PLGA molecular weight reduced DNA release rate [51]. Little or no release of DNA from PLGA particles was shown from PLGA microspheres where the PLGA molecular weight was greater than 30 kDa [52]. OligoDNA release results from PEI polyplexes in PLGA microparticles are consistent with previous results (Fig. 3). Only 3% of loaded oligoDNA, as a stable siRNA analog for this study, was released from the PLGA particles of molecular weight 57 kDa over 7 weeks in buffer. High molecular weight PLGA particles protect encapsulated DNA and maintain the particle integrity until particles are released from CPC to target cell candidates by CPC degradation. Clear differences are observed between particle degradation-release of nucleic acid payloads within cells (i.e., 12 days, Fig. 2G) versus that to water (negligible release after 50 days to water, Fig. 3) possibly facilitated by lysosomal degradation. siRNA
analogue/bPEI polyplex-loaded PLGA particles possess similar polyplex charge as RANK siRNA and the same particle surface charge when loaded into PLGA particles (Table 1). However, as expected, the actual siRNA/bPEI PLGA particles effectively reduced RANK expression in BMC cultures, compared with control siRNA analogue/bPEI-loaded PLGA particles, indicating that decreased RANK expression was due to specific siRNA taken-up by phagocytes and subsequent gene sequence-specific RANK message knockdown. The retention of physicochemical and functional integrity of RANK siRNA during the encapsulation also suggests that the polyplex polymer effectively protects siRNA during particle fabrication. Taken together, these results support the utility of siRNA/bPEI-loaded PLGA microparticles for specific suppression of RANK expression in osteoclast precursors in serum-based cultures of NP ratios of 20.

Studies have shown that cement scaffold microporosity largely improved bone in-growth by enhancing protein absorption to this enlarged surface area, promoting increased osteoblast attachment and improving microenvironmental cement solubility [53]. This concept is extended to PLGA microparticles encapsulating RANK siRNA within CPC as an injectable composite structural and drug delivery material. The design mandates particle-selective delivery to professional host-resident phagocytes responsible for bone loss in osteoporosis, and application of the CPC matrix as a local bone augmentation material adjacent to osteoporotic fixation. Addition of 20% (w/w) PLGA particles is the maximum loading to maintain composite cement injectability and retain all CPC physical parameters within surgically workable ranges [16,19,54,55], but properties of CPC containing 10% PLGA microspheres are closer to CPC alone [8]. Therefore, 10% (w/w) PLGA particle/CPC composite formulations were selected for siRNA delivery in this study. After cement curing and setting, the PLGA/CPC formulation formed a rigid scaffold with homogeneously distributed PLGA microspheres. Addition of these PLGA microparticles significantly increased the porosity of the cement (see Table 1), implying the improvement of interconnectivity within the cement. Significantly, as long as pore interconnectivity is obtained, PLGA particle release will not be dependent on CPC degradation or dissolution kinetics, yielding a sustained release system with some limited versatility based on particle loading. In addition, such interconnectivity also can accelerate overall aqueous phase access, penetration and degradation of CPC. Matrix mechanical properties could also be compromised with higher PLGA particle incorporation. Based on mechanical testing results from the 10% formulations of PLGA-loaded CPC, no significant differences were observed in stiffness between CPC alone and PLGA/CPC composites at their as-cured initial stage (See Fig. 4C).

For cell cultures, thin, flat CPC composite wafers were used to accelerate PLGA particle release to produce an experimentally accessible cell culture period. Successful knockdown of the RANK gene was evident in osteoclast precursor phagocytes with RANK siRNA/PEI PLGA particle-incorporated CPC. This proves the capability of this bone targeting delivery system to enter phagocytes and alter specific target message. Unchanged RANK expression observed from siRNA analogue/bPEI PLGA-incorporated CPC and blank CPC control cell cultures supports the desired specificity of knockdown by released siRNA.

We have previously shown specific siRNA-induced suppression of RANK expression resulted in significant inhibition of both osteoclast formation and also model bone resorption in both secondary RAW264.7 and primary cultures in vitro [34]. With this correlation, RANK expression was used here as the evaluation indicator for siRNA uptake and resulting phagocytosis inhibition. PLGA particle release must start with the particles exposed on the CPC matrix surface and the newly generated CPC pores then increase interconnectivity over time to facilitate further particle release and make the cement more porous and susceptible for further degradation. In vitro studies have shown that the overall medium pH for 10:90 PLGA/CPC samples decreased to pH 4.4 after 12 weeks’ incubation, ostensibly from PLGA acid by-product production. Significant pH reductions began after week 4 [8]. However, this in vitro release profile will be very different from in vivo kinetics, due to body fluid transport, bone mechanical stress influences, and individual pathological conditions. Local bone tissue systems could be buffered sufficiently, depending on the deployed CPC volume and the CPC-PLGA intrinsic weak acid-base interactions.

Future studies will exploit local siRNA in vivo delivery to osteoporotic bone models from bone augmentation devices and injected liquid depot. This strategy seeks to provide a clinically relevant approach to improve osteoporotic fragility fracture healing using current clinical bone augmentation strategies, while also remaining amenable to local siRNA therapeutic drug delivery directly from bone defects and common fracture fixation hardware. Many RNA targets are possible with this approach [27].

5. Conclusions

This study supports the dual concept of applying the unique combination of injectable bone-augmenting CPC and local siRNA cell-targeted PLGA particle delivery with siRNA cell uptake targeted to bone phagocytes from CPC matrices injected into bone augmentation sites. We show that siRNA encapsulated within PLGA microspheres does not significantly impair its functional integrity in target message knockdown in bone phagocytic cultures. As a result, targeted phagocytic cell-specific delivery can be achieved by controlling PLGA particle sizes within the micron range. RANK expression has been specifically knocked down in osteoclasts and their precursor phagocytic cells when delivered in several ways to cell cultures, resulting in inhibited bone resorption [34]. Phagocytosis-specific cell delivery of siRNA against RANK using particles selectively targets osteoclastic phenotypes. This is important in the context of treating bone augmentation zones where osteoporosis has produced low bone density and facilitates CPC augmentation injection. In addition, resulting PLGA particle release and implant degradation produces CPC macro pores known to facilitate further particle release, and promote bone in-growth, simultaneously with RANK siRNA release. This strategy is intended to both suppress bone resorption in peri-implant areas needed to facilitate healing and enhance mechanical stabilization processes for fixation of fragility fractures using clinically common bone augmentation strategies for local siRNA delivery to bone.

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