Intra-Articular Controlled Release of Anti-Inflammatory siRNA with Biodegradable Polymer Microparticles Ameliorates Temporomandibular Joint Inflammation

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Abstract

We investigated the in vivo therapeutic efficacy of an intra-articular controlled release system consisting of biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) microparticles (MPs) encapsulating anti-inflammatory small interfering RNA (siRNA), together with branched poly(ethylenimine) (PEI) as a transfecting agent, in a rat model of painful temporomandibular joint (TMJ) inflammation. The in vivo effects of PLGA MP dose and siRNA-PEI polyplex delivery were examined via non-invasive meal pattern analysis and by quantifying the protein level of the siRNA target as well as of several downstream inflammatory cytokines. Controlled release of siRNA-PEI from PLGA MPs significantly reduced inflammation-induced changes in meal patterns compared to untreated rats with inflamed TMJs. These changes correlated to decreases in tissue-level protein expression of the siRNA target to 20–50% of the amount present in the corresponding control groups. Similar reductions were also observed in the expression of downstream inflammatory cytokines, e.g., interleukin-6 (IL-6), whose tissue levels in the siRNA-PEI PLGA MP groups were 50% of the values for the corresponding controls. This intra-articular sustained release system has significant implications for the treatment of severe TMJ pain, and also has the potential to be readily adapted and applied to mitigate painful, chronic inflammation in a variety of conditions.

Keywords

Controlled drug release; drug delivery; microsphere; inflammation; immunomodulation

1. INTRODUCTION

Temporomandibular joint (TMJ) disorders are a common and heterogeneous group of diseases that cause painful, progressive joint degeneration that restricts daily activities, including talking and chewing [1]. In severe TMJ degeneration, oral medications are often insufficient for pain relief, and thus intra-articular injections of corticosteroids or hyaluronic

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Acid are necessary to treat pain. However, these intra-areolar formulations are complicated by rapid clearance of injected agents, which necessitates frequent injections that carry a high risk of iatrogenic injury [1, 2]. These limitations have motivated research into the development of novel therapeutics, including sustained release delivery systems, to alleviate severe TMJ pain.

Recently, inflammatory cell signaling via the immunoglobulin type G (IgG) cell-surface Fc receptor (FcγRIII, also known as CD16) has been implicated as a significant factor in painful TMJ inflammation [3]. FcγRIII is a cell-surface receptor on macrophages and neutrophils whose presence stimulates the secretion of several inflammatory cytokines [3]. Silencing of FcγRIII signaling via intra-articular injection of a solution of small interfering RNA (siRNA) targeting FcγRIII, using poly(ethyleneimine) (PEI) as a transfecting agent, showed promising results in a rat model of TMJ inflammation for two days [3]. However, as with the corticosteroid injections currently used in humans, a major challenge for intra-articular delivery of siRNA is the lack of a method for intra-articular sustained release [4, 5].

Motivated by these results, we have been developing an intra-articular controlled release system for the rat TMJ based on biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) microparticles (MPs). Several studies have demonstrated that PLGA MPs are effective for in vitro controlled release of siRNA [6–8], including sustained release of polyplexes consisting of siRNA together with the transfecting agent PEI [8], but few in vivo studies of siRNA release from PLGA MPs exist in the literature. One report has described the efficacy of PLGA-based MPs in delivering siRNA to treat tumors in vivo [7]. We have previously shown that non-drug-loaded PLGA MPs are biocompatible in vivo in the rat TMJ [9].

In a continued effort to develop an intra-articular controlled release system for the rat TMJ, this work reports the in vivo therapeutic efficacy of PLGA MPs loaded with anti-FcγRIII-siRNA-PEI polyplexes in a rat model of painful TMJ inflammation. We examined the in vivo effects of two factors, the presence (vs. absence) of siRNA-PEI polyplexes within the PLGA MP-based controlled release system, as well as the dose (low vs. high) of PLGA MPs injected into the TMJ. We hypothesized that siRNA and PEI would have distinct release kinetics from the PLGA MPs, but would immediately assemble into siRNA-PEI polyplexes upon release, become internalized by inflammatory cells in the TMJ tissue, and exert a therapeutic effect on painful TMJ inflammation by silencing the target gene, FcγRIII, and reducing the expression of downstream inflammatory cytokines. The specific questions addressed herein are: What is the effect of this intra-articular controlled release system on (1) inflammation-induced changes in rat meal parameters and (2) TMJ tissue expression of the target gene and related inflammatory cytokines? (3) What are the release kinetics of siRNA and PEI from the controlled release system? (4) Is the size of the siRNA-PEI polyplexes released over time consistently within the known limits for cellular uptake?

2. MATERIALS and METHODS

2.1 Experimental design

A full factorial design was used to evaluate the effect of two factors on TMJ inflammation. The two factors were the siRNA-PEI polyplexes and PLGA MP dose, and each factor had two levels: presence vs. absence of anti-inflammatory siRNA-PEI polyplexes, and low vs. high PLGA MP dose, respectively. Five groups were included in the design (see Figure 1). The two “anti-FcγRIII siRNA MPs” groups received polyplex-loaded PLGA MPs; one received a low dose of MPs (1.5 mg per joint), while the other received a high dose (2.3 mg per joint). The two “blank MPs” groups received the corresponding low and high amounts of empty MPs. All groups were injected with pro-inflammatory Complete Freund’s Adjuvant.
(CFA) to induce TMJ inflammation. A “CFA only” control group was included, which was not injected with MPs.

2.2 Meal pattern analysis

This study was approved by the Baylor College of Dentistry Institutional Animal Care and Use Committee, and conducted in accordance with the National Institutes of Health animal care and use guidelines. 45 healthy adult male Sprague-Dawley rats (Harlan Industries, Houston, TX) (250–300g) were randomly assigned to the five groups and housed individually in cages equipped with previously described photobeam computer-activated pellet feeders [3, 9]. Briefly, when a rat consumed a 45 mg pellet (Bioserv, Frenchtown, NJ), an infrared beam signaled the computer to record the event and dispense a new pellet.

After acclimating to the cages for several days, baseline meal parameters were recorded for 2 days, and then all rats except for the “CFA only” group were anesthetized using 5% v/v isoflurane in oxygen. PLGA MPs were suspended at 50 or 75 mg/ml in 10% v/v Tween 80 in normal saline, a carrier solution that has been previously shown to be biocompatible in the rat TMJ [9]. An experienced individual (P.R.K.) performed bilateral 30 μl intra-articular TMJ injections consisting of PLGA MP suspensions using an established approach that reliably delivers the MPs to the superior joint space of the TMJ [9]. 72h later, all rats were anesthetized and received bilateral 20 μl TMJ injections of 15 μg CFA (Chondrex, Redmond, WA) to induce TMJ inflammation [3]. Meal parameters were recorded for a total of 9 days, and then the rats were euthanized by decapitation. Of the 9 rats in each group, 6 were randomly chosen for ELISA and Western blot analysis, while the remaining 3 were reserved for immunohistochemistry. For ELISA and Western blot samples, the TMJ was dissected according to an established protocol [3], flash-frozen, and stored at −80°C. For immunohistochemistry, the TMJ was removed en bloc, fixed in 4% w/v paraformaldehyde in phosphate-buffered saline (PBS) for 48h, demineralized in 0.5 M EDTA using a Biowave® microwave (Pelco, Redding, CA), and then submerged in 25% w/v sucrose for 24h, as previously described [3, 9].

2.3 Immunohistochemistry

20 μm thick sagittal sections were cut using a cryotome (Damon/IEC, Needham Heights, MA) and placed on Superfrost Plus slides (StatLab, Lewisville, TX). The slides were blocked for 1h in PBS with 5% v/v goat serum and 0.3% v/v Triton X-100, and then incubated overnight at 4°C in PBS with 1% w/v bovine serum albumin, 0.3% Triton X-100, and 1:100 diluted anti-FcγRIII and anti-CD14 primary antibodies (cat. no. sc-20627 and sc-5749, respectively; Santa Cruz Biotechnology, Santa Cruz, CA). After 3 rinses (5 min each) with 0.3% v/v Triton X-100 in PBS, the slides were incubated for 1h at room temperature in the dark in PBS with 1% w/v bovine serum albumin, 0.3% Triton X-100, and 1:500 diluted secondary antibodies (Alexa Fluor 488 nm anti-rabbit and Alexa Fluor 546 anti-goat; Invitrogen, Carlsbad, CA). After three rinses in 0.3% v/v Triton X-100 in PBS, the slides were mounted with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA) and imaged using a fluorescence microscope (Nikon Instruments, Melville, NY).

2.4 Protein extraction, ELISAs, and Western blots

Each frozen TMJ sample was placed in 1ml of 1% v/v HALT protease inhibitor in TPER lysis buffer (Thermo Scientific, Waltham, MA), pulverized with a tissue homogenizer, and centrifuged at 700 × g at 4°C. The supernatant was removed and used for analysis. Total protein was quantified using a bicinechonic acid assay kit (Pierce Biotechnology, Rockford, IL). ELISA kits were used to quantify interleukin(IL)-6 (cat. no. R6000B, R&D Systems, Minneapolis, MN) and IL-1β (cat. no. KRC0011, Invitrogen). For Western blots, ~30 μg of protein together with LDS sample buffer and reducing agent (NuPAGE, Invitrogen) were
loaded into NuPAGE 4–12% Bis-Tris (1.0mm x 10 well) gels together with a protein ladder (1:1 mix of Novex Sharp and Magic Mark, Invitrogen). After 35 min, the gel contents were transferred to poly(vinylidene difluoride) membranes over 45 min.

Each membrane was incubated in antibody buffer (5% w/v dry milk in TBST (900 ml ultrapure (Type I) water (Super-Q Water Purification System, EMD Millipore, Billerica, MA), 100 ml 10x Tris-buffered saline, 1 ml Tween 20)) for 1h at room temperature with gentle agitation. After 3 rinses in 20 ml TBST (5 min each), the membrane was submerged in antibody buffer containing 1:500 diluted anti-FcγRIII primary antibody (Santa Cruz) with agitation overnight at 4°C. After equilibrating to room temperature, the membrane was rinsed 3 times with TBST and then incubated for 2h with agitation in antibody buffer containing 1:500 diluted secondary antibody (cat. no. PI-1000, Vector, Burlingame, CA). After 3 rinses in TBST and 2 rinses in 20 ml ultrapure water (2 min each), the membrane was covered with ECL Plus reagent (Amersham, Piscataway, NJ) and exposed to dental X-ray film (BX film, MIDSCI, St. Louis, MO). All membranes were stripped using ReBlot Plus Strong reagent (Millipore, Billerica, MA) for 15 min and the above procedure was repeated using a primary anti-β-actin antibody (cat. no. 4970, Cell Signaling, Danvers, MA) and a suitable secondary antibody (cat. no. 7074, Cell Signaling, Danvers, MA), both diluted 1:1000 in antibody buffer.

The X-ray films were scanned at 1200 dpi using a photo-quality scanner (V200, Epson, Long Beach, CA) with all automatic correction features (e.g., smoothing, descreening) disabled. Densitometry was performed using MCID software (MCID, Linton, Cambridge, England) according to an established protocol [10].

### 2.5 Polyplex preparation

The two siRNA sequences (siRNA1 sense: 5′-CCAGCUCUCUAGUGUGGUUTT-3′ and siRNA2 sense: 5′-GCUCUCUAGUGGUUCCATT-3′; Silencer® siRNA ID no. 195781 and 195782, respectively; Ambion, Austin, TX) were selected based on a prior study where a 1:1 mixture of “free” siRNA1-PEI and siRNA2-PEI had a significant effect on TMJ inflammation for 48h [3]. Each siRNA sequence was reconstituted at a concentration of 3.2 nmol siRNA/μl in nuclease-free water according to the manufacturer’s instructions. For each siRNA type, 25 μl of stock were added to 30 μl of a 60 mg/ml solution of branched PEI (nominal weight-average molecular weight 25 kDa, Aldrich, Milwaukee, WI) in nuclease-free water. The mixture was vortexed for 5 s, incubated at room temperature for 20 min to allow for polyplex formation, and then immediately used to prepare (siRNA-PEI)-loaded PLGA MPs. The amount of siRNA and PEI was selected to achieve a 16:1 Nitrogen:Phosphate (N:P) ratio, calculated as previously described [8, 11] (see Table II footnote).

For polyplexes with rhodamine-labeled PEI (r-PEI), 2 mg/ml PEI in nuclease-free PBS was first conjugated to rhodamine dye using a commercial kit (cat. no. 53031, Pierce). To remove excess salt, the r-PEI solution was loaded into dialysis cassettes (cat. no. 66203, Pierce) and dialyzed against a 200× larger volume of: 1× PBS for 1h, 0.5× PBS for 8h, and then nuclease-free water (twice, each for 2h). Dialysis was performed at 4°C in the dark to prevent quenching of the rhodamine. All materials, including the cassettes, were treated with RNase Zap (Ambion) to inactivate RNases that might later result in siRNA degradation. Following dialysis, the r-PEI solution was flash-frozen and then lyophilized overnight in dark conditions. The r-PEI was dissolved at 60 mg/ml in nuclease-free water and used to prepare siRNA-r-PEI polyplexes. A single r-PEI batch was used for all experiments.
2.6 PLGA MP preparation and characterization

The MPs consisted of a physical blend of 5% w/w poly(ethylene glycol) (PEG; nominal molecular weight of 4600; Aldrich, Milwaukee, WI) in PLGA with a copolymer ratio of 50:50 (Lakeshore Biomaterials, Birmingham, AL). The PLGA had a number-average molecular weight of 42500 ± 1600 Da and a polydispersity index of 1.53 ± 0.03, determined via gel permeation chromatography (n = 3 samples; Phenogel Linear Column, Phenomenex, Torrance, CA; Differential Refractometer 410, Waters, Milford, MA) using a polystyrene standard curve (Fluka, Switzerland). PLGA MPs were synthesized using an established double emulsion solvent extraction technique [8, 9, 12]. Briefly, the aqueous polyplex solution was added to a mixture of PLGA and PEG dissolved in dichloromethane, vortexed for 1 min, and then added to an aqueous solution with 0.15% w/v poly(vinyl alcohol) and 1% v/v isopropanol. Each MP formulation contained siRNA-PEI or siRNA-r-PEI polyplexes with a single siRNA sequence (i.e., either siRNA1 or siRNA2). MPs loaded with r-PEI were prepared in foil-wrapped beakers using indirect lighting to prevent quenching of the dye. All aqueous solutions were prepared with nuclease-free water (W3450, Teknova, Hollister, CA) and all glassware was treated with RNAse Zap (Ambion) to prevent siRNA degradation. Following fabrication, all MPs were flash-frozen in liquid nitrogen, lyophilized for 48h, and then stored under a nitrogen atmosphere at −20°C in a non-defrosting freezer to prevent degradation. MP diameter was determined using a Multisizer3 Coulter Counter (Beckman Coulter).

2.7 Quantification of entrapment efficiency and polyplex release

15.0 mg of each MP formulation were incubated in 0.5 ml dichloromethane at room temperature until completely dissolved (~20 min). 0.5 ml nuclease-free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) were added; the vial was vortexed for 1 min, centrifuged at 11,000 × g for 5 min at 4°C, and then the aqueous phase was removed as previously described [8, 11]. The extraction was repeated, the two 0.5 ml aqueous phases were combined, and then siRNA or r-PEI content was measured.

To quantify release, 15.0 mg samples (n = 4 from each MP formulation) were suspended in 0.5 ml nuclease-free PBS (pH 7.4) and incubated at 37°C with agitation. At each timepoint (2h, 4h, 8h, 12h, and days 1, 2, 3, 4, 8, 11, 14, 17, 21, 25, 28): the vials were centrifuged at 11,000 × g for 5 min at 4°C; the supernatant was replaced with fresh PBS; and the release vials were returned to 37°C. Collected supernatants were immediately stored at −20°C in a non-defrosting freezer for later analysis.

2.8 Quantification of siRNA and r-PEI content

To measure siRNA, 50 μl of each release sample or standard were combined with 100 μl of 0.5% v/v PicoGreen dye (Invitrogen) in TE buffer (pH 12) and analyzed with a fluorescence plate reader (excitation/emission wavelength = 485/530 nm) using an established protocol [8]. To measure r-PEI, 100 μl of each sample or standard was added to a well of an opaque 96-well plate and then r-PEI content was quantified using a fluorescence plate reader (excitation/emission wavelength = 530/620 nm) according to an established protocol [8, 13]. The standard curve consisted of siRNA-PEI or siRNA-r-PEI polyplexes with 16:1 N:P and the same siRNA sequence (i.e., either siRNA1 or siRNA2) as the samples. All samples and standards were run in triplicate.

2.9 Dynamic light scattering

DLS was performed using a 90PLUS particle size analyzer (Brookhaven Instruments, Holtsville, NY) at 659 nm wavelength and 37°C. Within the BIC software of the DLS apparatus, the accumulated autocorrelation function was analyzed via the non-negatively
constrained least squares algorithm to derive the intensity-weighted particle size distribution. The DLS results of the release samples were compared to those of freshly prepared polyplexes to identify the polyplex size, as previously described [8]. For each siRNA type, polyplexes were prepared with N:P ratios spanning the range of those observed in the release samples, incubated at 37°C, and then their diameter was measured via DLS after 4h and again at 24h.

2.10 Statistics

Meal pattern data were analyzed using a repeated measures analysis of variance (p < 0.05) to examine differences amongst groups over time. Multiple pair-wise comparisons were made via Bonferroni post hoc analysis at 95% confidence. Meal pattern data are reported as mean ± standard deviation for n = 9 rats per group. The data are expressed as a percentage of the average pre-MP-injection value; this method eliminates inherent differences amongst the groups, clarifying differences due to the treatments [14].

For both ELISA and Western blot data, differences amongst groups were determined via one-way analysis of variance (p < 0.05) followed by Bonferroni post hoc analysis (p < 0.05). This method was also used to compare the diameters of the freshly prepared polyplexes to those of the release samples with equivalent N:P. ELISA data are reported as mean ± standard error of the mean for n = 6 rats per group. The value for each sample is the amount of cytokine divided by the amount of total protein. Similarly, the Western blot densitometry values are the ratio of FcγRIII to β-actin. The data are reported as mean ± standard deviation for n = 6 rats per group.

Release and hydrodynamic diameter data were analyzed using a repeated measures analysis of variance (p < 0.05), followed by Bonferroni post hoc analysis (p < 0.05). The release values for each MP formulation are a percentage of the total measured amount of siRNA or r-PEI entrapped. Reported release values and hydrodynamic diameters represent the mean ± standard deviation for (n = 4) samples at each timepoint. Entrapment efficiencies are presented as mean ± standard deviation (n = 3 samples per MP formulation). MP diameters are reported as mean ± standard deviation of n = 1500 particles per MP batch.

3. RESULTS

3.1 Meal Pattern Analysis

Figure 1 summarizes the effect of anti-inflammatory anti-FcγRIII-siRNA-PEI polyplexes (polyplexes present or absent) and PLGA MP dose (low or high) on nocturnal meal duration (Figure 1a,b) and food intake (Figure 1c,d) of rats with TMJ inflammation. TMJ inflammation was induced in all groups via bilateral intra-articular injection of CFA. In the “CFA only” control group, no MPs were injected. All groups differed significantly (p < 0.05) in terms of meal duration (Figure 1a,b). All groups except the CFA only control and High blank MPs group differed significantly (p < 0.05) in terms of food intake (Figure 1c).

Prior to the induction of inflammation (days 2–5), the presence of MPs did not have any significant irritating effect on either meal parameter. Following CFA injection, siRNA-PEI-loaded PLGA MPs significantly (p < 0.05) reduced inflammation-induced changes in meal duration (Figure 1a,b), with the Low anti-FcγRIII siRNA MPs group having the most therapeutic effect. At the same time, the food intake of siRNA-PEI-loaded MP groups approached baseline sooner than the control groups (Figure 1c,d). This reduction in inflammation-induced changes in food intake was statistically significant (p < 0.05), with the High anti-FcγRIII siRNA MPs group having the most therapeutic effect (Figure 1c).
3.2 Inflammatory Cytokine and FcγRIII Expression

Figure 2 shows the protein levels of two inflammatory cytokines, IL-6 and IL-1β, whose secretion is stimulated by FcγRIII. Anti-FcγRIII-siRNA-PEI-loaded PLGA MPs significantly (p < 0.05) reduced IL-6 levels compared to all other groups. The Low anti-FcγRIII siRNA MPs also significantly reduced IL-1β levels (p < 0.05), a finding consistent with the meal duration findings (Figure 1a,b), where this group had a greater therapeutic effect than the higher dose of siRNA-PEI-loaded MPs. The High blank MPs groups had slightly higher (p < 0.05) IL-6 levels than all other groups, including the CFA only control. This elevated inflammatory cytokine value for this group is consistent with the meal duration data, where the High blank MPs had higher meal duration over days 7–9 compared to all other groups, suggesting a higher degree of painful inflammation.

The expression of the siRNA target, FcγRIII, shown in Figure 3a, mirrored the IL-6 levels (Figure 2), as well as the meal duration data (Figure 1a,b). The High blank MPs had significantly elevated FcγRIII values (p < 0.05) compared to all other groups, while the siRNA-PEI-loaded MP groups had lower FcγRIII levels than all other groups, although the effect of the siRNA-PEI-loaded MPs was not significant in this case (p > 0.05). A representative Western blot is shown in Figure 3e, which indicates several bands for FcγRIII, including some outside the expected size range. All bands shown were included in the densitometry because larger bands (~160 kDa) likely represented complexes of FcγRIII and other species, while the smaller bands (~40 kDa) were consistent with FcγRIII subunits [15].

Qualitative analysis of immunostaining for FcγRIII was also consistent with the Western blot densitometry. Representative immunostained images from a rat in the High anti-FcγRIII siRNA MPs group are shown in Figure 3. Since siRNA silences the protein level expression of its target, and these images are from the TMJ of a rat treated with siRNA-PEI, it would be expected that the tissue level expression of the siRNA target would be reduced. Indeed, most of the inflammatory cells in the tissue did not express the siRNA target, FcγRIII. Cells expressing FcγRIII were mainly observed within and near blood vessels, suggesting that they were newly arrived to the site of the inflammation.

3.3 Release of siRNA and PEI from PLGA MPs

Table I summarizes the entrapment efficiency of siRNA and PEI within the PLGA MPs, as well as MP diameter. There were no statistically significant differences (p > 0.05).

Although we have previously reported on the release of siRNA-PEI polyplexes from PLGA MPs [8], release studies were included in this work because the siRNA sequences used herein, as well as the initial siRNA and PEI loading values (Table I), were notably different from those previously reported. Figure 4 depicts the cumulative release of siRNA and rhodamine-labeled PEI (r-PEI) from the MPs. Although MP formulations containing siRNA1-PEI and siRNA2-PEI polyplexes were mixed in a 1:1 ratio prior to intra-articular injection, the MPs were evaluated separately for the in vitro release studies. This enabled siRNA and r-PEI quantification via comparison to a standard curve derived from polyplexes containing the identical siRNA sequence. Both siRNA1 and siRNA2 target FcγRIII; the two sequences differ by a frame-shift of three nucleotides (see Methods section 2.5).

The siRNA sequence did not significantly affect either siRNA or r-PEI release (p > 0.05; Figure 4). Both siRNA and r-PEI showed an initial burst release for 24h. Approximately 60–65% of the encapsulated siRNA (Figure 4a), and ~20% of the encapsulated r-PEI (Figure 4b), was released during this burst period. This was followed by a lag period for siRNA, after which release resumed around day 8. In contrast, r-PEI burst release was followed by slow linear release throughout the remainder of the 28 day release period. In contrast to the...
encapsulated siRNA, which was almost completely (>90%) released after 28 days, only ~30% of the encapsulated PEI was released by day 28. At each timepoint, the siRNA and r-PEI release data were used to calculate the N:P ratio, i.e., the ratio of protonatable amine (N) groups to nucleic acid backbone phosphates (P), as summarized in Table II. The N:P ratios of the polyplexes released from the two MP formulations did not differ over time (p > 0.05).

3.4 Size of siRNA-PEI released from PLGA MPs

Figure 5 illustrates the average diameter of the polyplexes released from the siRNA1-PEI-loaded and the siRNA2-PEI-loaded MPs at each timepoint over the 28 day study. Although the average diameters of the polyplexes for each siRNA type were similar when considering all timepoints together (135 ± 50 nm for siRNA1-PEI and 117 ± 50 nm for siRNA2-PEI), the size of the two polyplex types at each timepoint differed significantly (p < 0.05), based on repeated measures analysis of variance.

The peaks in the dynamic light scattering (DLS) output corresponding to the polyplexes were identified by preparing fresh siRNA1-PEI and siRNA2-PEI polyplexes with identical N:P ratios and comparing these to the DLS data. Representative DLS curves from each timepoint of the release study are shown in Figure 6. Most timepoints contained peaks for both siRNA1-PEI and siRNA2-PEI that did not differ significantly (p > 0.05) from freshly prepared polyplexes (orange arrows in Figure 6). However, several timepoints had no detectable polyplexes, as shown by the white-filled data points at 0 nm in Figure 5. For instance, only free PEI was detected for siRNA2-PEI on day 3, as shown in Figure 6. The yellow arrows indicate peaks that were statistically different from pure polyplexes with the same N:P ratio (p < 0.05). Some larger (500–1000 nm diameter) particles were observed at several timepoints; generally, their diameter did not differ (p > 0.05) from that of the PLGA MP derivatives observed in the release medium of blank MPs analyzed via DLS (gray shading in Figure 6). Additionally, several of the early timepoints contained small particles (i.e., of diameter <10 nm) that did not differ in size (p > 0.05) from free PEI (green arrows in Figure 6).

4. DISCUSSION

This study demonstrated that intra-articular controlled release of anti-FcγRIII-siRNA-PEI polyplexes from PLGA MPs ameliorates in vivo TMJ inflammation, as quantified by changes in meal patterns and protein expression of pro-inflammatory signals. Although we have previously established the intra-articular biocompatibility of 15–30 mg/ml of empty PLGA MPs in healthy, non-inflamed rat TMJs [9], to our knowledge, this is the first study to examine the in vivo effect of siRNA-PEI-loaded PLGA MPs in an inflamed TMJ model. The higher dose of PLGA MPs (75 mg/ml) used in this study exceeds the previously evaluated PLGA MP dosages. This concentration was selected to increase the dose of siRNA-PEI delivered to the rat TMJ, with the goal of improving therapeutic efficacy.

Remarkably, compared to the lower dose of siRNA-PEI-loaded PLGA MPs, the higher dose of siRNA-PEI-loaded MPs had reduced therapeutic efficacy. Following CFA injection, the High blank MPs increased meal duration (p < 0.05) more than CFA only (Figure 1a). This finding, together with the significantly higher (p < 0.05) IL-6 levels and FcγRIII expression of the High blank MP group compared to both the Low blank MPs and the microparticle-free CFA only control (Figures 2a,3a), suggests that the higher MP dose increased TMJ irritation. Notably, although the higher dose of blank MPs caused a significant increase in IL-6 and FcγRIII, IL-1β levels were not elevated. This combination suggests that the mechanism of irritation by the high dose of blank MPs involves toll-like receptor (TLR) activation.
TLRs are transmembrane proteins that serve as a component of innate immunity, but have also been implicated in particle-induced inflammation associated with biomaterials [16–18]. The significant increase in IL-6 and FcγRIII, but not IL-1β (Figure 2,3a), by the higher dose of blank MPs is consistent with TLR activation. IL-1β and IL-6 were selected for quantification because they are elevated in the TMJs of patients and animal models with painful inflammation [19–21]. TLR stimulation by microparticles has been shown to trigger IL-6-mediated inflammation [17]. Additionally, there is significant structural and functional cross-talk between the TLR and FcγRIII pathways [15], which suggests a mechanism for both the increased FcγRIII expression in the High blank MP group, and the corresponding reduction seen with the anti-FcγRIII-siRNA-PEI-loaded PLGA MPs (Figure 3a). It is possible that higher dose of PLGA MPs (75 mg/ml) surpassed a threshold for TLR activation, while the lower dose (50 mg/ml) did not have this effect. Future studies of MP-based delivery systems for the TMJ should include quantification of TLR expression to further evaluate this proposed mechanism.

The small dip in food intake seen after MP injection (Figure 1c,d) for all groups except the “CFA only” control, which did not receive any injection on day 2, likely represents a reaction to the minor trauma caused by the needle used for the injection. The magnitude of the change is consistent with previously reported data using empty PLGA MPs as well as injections of the carrier solution alone [9]. In all cases, this change was not statistically significant (p > 0.05).

The two anti-FcγRIII siRNA sequences used in this study were selected based on a previous study in the rat TMJ model indicating that intra-articular injection of a 1:1 mixture of “free” siRNA1-PEI and siRNA2-PEI had a significant effect on TMJ inflammation for 48h [3]. The mechanism of the anti-FcγRIII siRNA delivered in this study, as with all siRNAs, is to silence a specific gene by binding to messenger RNA and blocking protein translation [22, 23]. To correlate the therapeutic effects observed via meal pattern analysis with the known RNA interference mechanism of siRNA, TMJ tissue expression of FcγRIII was measured via Western blot densitometry (Figure 3). The results are qualitatively consistent with previously reported Western blot data derived from TMJs injected with anti-FcγRIII-siRNA-PEI polyplexes [3]. However, those results were not analyzed via densitometry.

FcγRIII stimulates secretion of several inflammatory cytokines, including IL-6 and IL-1β. Compared to previous measurements in the rat TMJ model [3, 24], IL-6 levels in this study were ~5× higher, while IL-1β levels were ~7× lower (Figure 2). Although decreased, IL-1β levels still exceeded the reported baseline values in the non-inflamed rat TMJ, ~0 pg IL-1β/mg total protein [24], indicating that TMJ inflammation had not yet subsided.

The evaluation of the PLGA MP-based controlled release formulations described in this work was limited by the relatively short duration of CFA-induced inflammation in the rat model [25]. We used CFA, a severely irritating water-in-oil emulsion containing inactivated Mycobacterium tuberculosis and paraffin oil, because this is an established, optimized method of creating a model of TMJ inflammation [14, 25–29]. The therapeutic effect of the siRNA-PEI-loaded PLGA MPs was evaluated via computerized meal pattern analysis, a non-invasive technique whose development was inspired by the painful, impaired eating in human TMJ disorder patients. This method has been used to evaluate various analgesic agents in the TMJ model [3, 14, 25, 27–29]. Although the PLGA MP formulations described in this work, as well as similar PLGA MPs with lower siRNA-PEI loading described elsewhere [8], can deliver siRNA-PEI polyplexes for up to 28 days, we selected day 9 as the endpoint so that the study could end prior to the complete resolution of TMJ inflammation, and thus differences in protein expression of inflammatory cytokines and FcγRIII might still be present. Future studies will need to address this limitation through the selection of a model with long-lasting TMJ inflammation.
The siRNA-PEI polyplexes released from PLGA MPs were within the established range (0–150 nm) for intracellular uptake and subsequent gene silencing [30, 31]. This is consistent with the observed in vivo efficacy of the siRNA-PEI-loaded PLGA MPs in alleviating TMJ inflammation.

During the in vitro release study, free PEI was detected via DLS at several timepoints (green arrows in Figure 6), in contrast to a previous study of siRNA-PEI-loaded PLGA MPs [8], in which free PEI was almost never detected in the release medium. In that study, the absence of free PEI was presumed to reflect the binding of the cationic PEI with anionic PLGA MP derivatives [8]. The 16× greater amount of PEI used in this study may have outweighed the number of anionic molecules present. Free PEI is highly cytotoxic [30, 32–34] and thus it is possible that the improved therapeutic efficacy of the lower dose of siRNA-PEI-loaded MPs may reflect the reduced amount of free PEI present. However, it is more likely that the higher amount of MPs was responsible for the added joint irritation, due to the significant increase (p < 0.05) in meal duration, IL-6 levels, and FcγRIII expression seen with the High blank MPs compared to all other groups (Figures 1a, 2a, 3a). This is further supported by a recent study involving the intra-articular injection of a much larger dose of “free” siRNA-PEI polyplexes into the rat TMJ, where no adverse effect was seen compared to injection of plain siRNA [3]. In fact, inflammation-induced changes to meal duration were further reduced with siRNA-PEI delivery.

CONCLUSIONS

We have demonstrated that intra-articular injection of anti-FcγRIII-siRNA-PEI-loaded PLGA MPs has a therapeutic effect on in vivo TMJ inflammation, as indicated by changes in meal patterns and protein expression of pro-inflammatory signals. The siRNA-PEI-loaded PLGA MP formulations showed sustained release of siRNA and PEI over 28 days in vitro, although the pattern of siRNA release differed from that of PEI. The diameter of the siRNA-PEI polyplexes released from the PLGA MPs was consistently within the established range for efficient intracellular uptake. In a week-long in vivo study in a rat TMJ model, the siRNA-PEI-loaded MPs reduced inflammation-induced changes in meal parameters and inflammatory cytokine expression. To our knowledge, this is the first study to evaluate, and demonstrate the efficacy of, an intra-articular controlled release system in an inflamed TMJ model. This novel intra-articular controlled release system not only has the potential to greatly improve TMJ therapeutics, but can be readily adapted and used to mitigate painful tissue damage in various other inflammatory conditions.

Acknowledgments

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Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>CD16</td>
<td>Fc receptor-III for IgG</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s Adjuvant</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>FcγRIII</td>
<td>alternative abbreviation for CD16</td>
</tr>
<tr>
<td>IgG</td>
<td>type G immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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</tbody>
</table>

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References


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Figure 1. Impact of siRNA-PEI-loaded MPs on meal parameters
Anti-FcγRIII-siRNA-PEI-loaded PLGA MPs reduced inflammation-induced changes in both (a,b) meal duration and (c,d) food intake. Dashed lines indicate: the time of MP injection for all groups (except “CFA only,” which did not receive MPs), and the time that pro-inflammatory CFA was injected. Letters A-E and F-I indicate groups that differ significantly ($p < 0.05$) from each other in panels (a,b) and (c,d), respectively. For each pair of panels, groups marked with the same letter were not statistically different ($p > 0.05$). Note that for both meal duration and food intake data, the CFA only group is shown in both panels (a,b) and (c,d), respectively. Values for each group are expressed as a percentage of the average pre-injection value. Data points represent the mean ± standard deviation for $n = 9$ rats. Error bars are included for all groups, though they are too small to resolve in some cases.
Figure 2. Effect of siRNA-PEI-loaded MPs on cytokine levels

Anti-FcγRIII-siRNA-PEI-loaded PLGA MPs reduced protein-level expression of inflammatory cytokines interleukin-6 (IL-6) and IL-1β. Protein was extracted from retrodiscal TMJ tissue on day 9 of the experiment, and then interleukin levels were measured via ELISAs. Values for each group are expressed relative to the total protein content; data points represent the mean ± standard error of the mean for *n* = 6 rats. In each panel, groups marked with * differ significantly from all other groups, while pairs of groups marked with ** or *** differ significantly from all other groups but not each other (*p* < 0.05).
Figure 3. Effect of siRNA-PEI loaded MPs on target protein expression

Protein-level expression of the siRNA target, FcγRIII, was measured via Western blot densitometry using protein extracted from the retrodiscal TMJ tissue on day 9 of the study. In (a), values for each group are expressed relative to the value of β-actin, also measured via Western blot densitometry; data points represent the mean ± standard deviation for n = 6 rats. A representative Western blot for FcγRIII, including samples from each group, is shown in (e). Note that two High blank MP samples happen to have been run in sequential lanes as shown in (e); this is of no significance. The β-actin loading controls shown for each lane were obtained after the membrane was stripped and incubated with the appropriate antibodies as described in Methods 2.4. The molecular weights (kDa) shown at left were derived from a protein standard ladder included on the gel. Panels (b–d) show representative immunostaining for FcγRIII (i.e., CD16) expression in the TMJ of a rat given anti-FcγRIII-siRNA-PEI-loaded PLGA MPs. Most of the cells in the tissue did not express FcγRIII. FcγRIII-positive cells were localized near blood vessels (b,c), suggesting that they were newly arrived to the site of the inflammation. The positive control (CD14) is present on inflammatory cells (c,d) that would normally also express the siRNA target, FcγRIII. The scale bar indicates 50 μm and applies to images (b–d).
Figure 4. Release of siRNA and PEI from PLGA MPs

PLGA MP formulations encapsulating polyplexes consisting of the two different siRNA sequences, i.e., siRNA1-PEI and siRNA2-PEI polyplexes, had statistically equivalent (a) siRNA and (b) PEI cumulative release profiles (p > 0.05). Cumulative siRNA release (a) followed a triphasic pattern consisting of an initial burst release for 24h, followed by a lag period, after which release resumed around day 8. The PEI profiles (b) also had a 24h burst release period, followed by gradual, nearly linear PEI release until day 28. MPs for siRNA measurements were loaded with siRNA-PEI polyplexes, while MPs for PEI measurements were loaded with polyplexes containing rhodamine-labeled PEI (r-PEI). This was necessary because the fluorometric assays for siRNA and r-PEI had overlapping emission wavelengths. Release is expressed as a percentage of the amount of siRNA or PEI initially encapsulated (= entrapment efficiency × theoretical loading from Table I). Data points represent the mean ± standard deviation for n = 4 samples. Error bars are included for all data points, though they are too small to resolve.
Although the average diameters of the polyplexes for each siRNA type were similar when considering all timepoints together (135 ± 50 nm for siRNA1-PEI and 117 ± 50 nm for siRNA2-PEI), the size of the two polyplex types at each timepoint differed significantly ($p < 0.05$), based on repeated measures analysis of variance. Data are presented as the mean ± standard deviation for ($n = 4$) samples at each timepoint. Error bars are included for all data points, though they are too small to resolve in some cases. The white-filled data points at 0 nm correspond to groups with no detectable polyplexes (e.g., only free PEI was detected for siRNA2-PEI on day 3, as shown in Figure 6).
Figure 6. Size of materials released from PLGA MPs over time
Representative DLS curves at 37°C for (top row) pure PEI, pure siRNA1, pure siRNA2, and (remaining rows) particles released from each PLGA MP formulation at selected timepoints. The green arrows indicate peaks that did not differ significantly ($p > 0.05$) from pure PEI. The orange arrows indicate peaks that did not differ significantly ($p > 0.05$) from freshly prepared polyplexes of the same N:P ratio. The yellow arrows indicate peaks that differed significantly ($p < 0.05$) from freshly prepared polyplexes of the same N:P ratio. The gray bars span a range equivalent to the average hydrodynamic diameter ± standard deviation of particles observed in ($n = 3$) empty PLGA MP samples at each timepoint.
Table I
Entrapment efficiency of siRNA and PEI in the PLGA MPs

<table>
<thead>
<tr>
<th>MP contents</th>
<th>PLGA MP yield (%)$^a$</th>
<th>Theoretical loading (μg/mg PLGA MPs)$^b$</th>
<th>Entrapment efficiency (% of theoretical loading)$^c$</th>
<th>MP diameter (μm)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>siRNA1-PEI polyplexes</td>
<td>64 ± 5</td>
<td>siRNA: 7.4 ± 0.5</td>
<td>siRNA: 85 ± 12</td>
<td>18 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEI: 11.0 ± 1.0</td>
<td>PEI: 76 ± 7</td>
<td></td>
</tr>
<tr>
<td>siRNA2-PEI polyplexes</td>
<td>68 ± 5</td>
<td>siRNA: 6.1 ± 0.3</td>
<td>siRNA: 91 ± 11</td>
<td>18 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PEI: 13.0 ± 1.0</td>
<td>PEI: 78 ± 5</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>83 ± 2</td>
<td>none</td>
<td>none</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

$^a$ Final weight of PLGA MPs as a percentage of the starting amount of PLGA and PEG (248 mg per batch). Values represent average ± standard deviation of $n = 3$ batches.

$^b$ Calculated by dividing the amount of either siRNA (1100 μg) or r-PEI (2000 μg) added during synthesis by the actual weight of PLGA MPs from that batch.

$^c$ Entrapment efficiency of either siRNA or r-PEI calculated from: (amount extracted from 15 mg PLGA MPs)/(theoretical loading) × 100%.

$^d$ Determined via Coulter Counter analysis of ($n = 1500$) MPs per batch. Values shown are average ± standard deviation of ($n = 3$ batches) for each MP type.
### Table II

<table>
<thead>
<tr>
<th>Day</th>
<th>siRNA1-PEI-loaded PLGA MPs</th>
<th>siRNA2-PEI-loaded PLGA MPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2 ± 0&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>0.2</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>0.3</td>
<td>3 ± 1</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>0.5</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>1</td>
<td>1 ± 0</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>0 ± 0</td>
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</tr>
<tr>
<td>3</td>
<td>7 ± 1</td>
<td>9 ± 2</td>
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<tr>
<td>4</td>
<td>14 ± 2</td>
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<td>8</td>
<td>38 ± 8</td>
<td>34 ± 7</td>
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<tr>
<td>11</td>
<td>2 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>0 ± 0</td>
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<tr>
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<tr>
<td>21</td>
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<tr>
<td>25</td>
<td>0 ± 0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>28</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> N:P ratios expressed with P set to 1. For instance, an N:P of 2:1 is expressed as “2.”

<sup>b</sup> Calculated from the actual amount of siRNA and PEI detected from (n = 4) PLGA MP release samples for each group at each timepoint, as previously described [8]. To calculate the moles of phosphate (P) for the N:P ratio, the amount of siRNA was divided by its molecular weight (provided by the manufacturer) and then multiplied by 42 (since the siRNA used was double-stranded and 21 base pairs in length, yielding 42 phosphates per siRNA molecule). The moles of nitrogen (N) for the N:P ratio were approximated by dividing the amount of PEI by the molecular weight of the monomer (C<sub>2</sub>H<sub>5</sub>N) of linear PEI and then multiplying by 1 (since there is 1 mol of N per mol of monomer). The resulting N:P ratio is expressed as mean ± standard deviation for (n=4) siRNA and PEI measurements.