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Harnessing Inflammatory Signaling to Promote Bone Regeneration and Mitigate Joint Damage (Volume II)

by

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Background: Emerging Intra-Articular Drug Delivery Systems for the Temporomandibular Joint

Abstract: Temporomandibular joint (TMJ) disorders are a heterogeneous group of diseases that cause progressive joint degeneration leading to chronic pain and reduced quality of life. Both effective pain reduction and restoration of TMJ function remain unmet challenges. Intra-articular injections of corticosteroids and hyaluronic acid are currently used to treat chronic pain, but these methods require multiple injections that increase the risk of iatrogenic joint damage and other complications. The small and

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emerging field of TMJ tissue engineering aims to reduce pain and disability through novel strategies that induce joint tissue regeneration. Development of methods for sustained, intra-articular release of growth factors and other pro-regenerative signals will be critical for the success of TMJ tissue engineering strategies. This review discusses methods of intra-articular drug delivery to the TMJ, as well as emerging injectable controlled release systems with potential to improve TMJ drug delivery, to encourage further research in the development of sustained release systems for both long-term pain management and to enhance tissue engineering strategies for TMJ regeneration.

7.1. Introduction

Temporomandibular joint (TMJ) disorders are the main cause of chronic facial pain and a major cause of disability. Treatment of these disorders in the United States has an estimated cost of $4 billion per year (194). Unlike other degenerative joint diseases, which are more common in the elderly, TMJ disorders affect up to one-third of adolescents and young adults. The chronic pain associated with progressive TMJ degeneration limits talking, chewing, and other basic daily activities (195, 196). Current treatments for TMJ disorders are limited. In severe cases, both effective pain reduction and restoration of TMJ function remain an unmet challenge (197).

One main type of TMJ disorder is an osteoarthritis-like degenerative joint disease characterized by progressive bone and cartilage destruction and subsequent inflammation, which exacerbates joint tissue catabolism (12). Consequently, pharmacologic approaches to TMJ disorders have paralleled those for symptomatic treatment of osteoarthritis, including non-steroidal anti-inflammatory drugs (NSAIDs) and intra-articular injections
of either steroids or hyaluronic acid into the superior joint space (see Figure 7.1). However, use of these agents remains controversial in light of decades of mixed reports of intra-articular injections either accelerating TMJ destruction or triggering regeneration (198). As in the case of osteoarthritis, no agents are available to reverse the underlying TMJ disease. Consequently, current pain reduction techniques are effective in the early stages of the disease, but fail to alleviate the severe, chronic pain caused by advanced joint degeneration (12, 199).

There is a need for sustained release agents that effectively reduce pain and have minimal systemic side effects, enabling long-term administration without the disastrous ectopic effects seen with NSAIDs like rofecoxib (Vioxx®) (200). This review discusses methods of intra-articular drug delivery to the TMJ, as well as emerging injectable controlled release systems with potential to improve TMJ drug delivery, to encourage further research in the development of sustained release systems for both long-term pain management and to enhance tissue engineering strategies for TMJ regeneration.

7.2. Current Methods of Intra-Articular Injection

A variety of injectable corticosteroid and hyaluronic acid formulations are used to reduce the persistent pain associated with TMJ destruction. Localized drug delivery via intra-articular injections minimizes ectopic effects while alleviating joint pain and other symptoms. Although the Food and Drug Administration (FDA) has only approved intra-articular hyaluronic acid formulations for osteoarthritis of the knee, these formulations are still used to treat pain in a number of other joints, including the TMJ (12, 201).
Figure 7.1 Schematic of temporomandibular joint anatomy.
Schematic depicting the temporomandibular joint (TMJ), indicated by the red box in the top panel. The lower panel shows relevant components of joint anatomy, including the TMJ disc, the head of the mandibular condyle, and a portion of the connective tissue capsule that envelops the joint. The superior joint space is also specifically indicated, and it is into this space that intra-articular TMJ injections are made.
Studies of the efficacy of intra-articular TMJ injections have shown mixed results, with improvement in some patients and disease progression in others (198). Alarming reports of post-injection complications, including cartilage destruction, bone necrosis, and progression of joint disease, have discouraged their use for TMJ pain (202, 203). These reports often describe isolated patients given repeated intra-articular injections (203-205). High doses of corticosteroids are known to increase the risk of aseptic bone necrosis. In one case report, a previously asymptomatic patient with TMJ inflammation developed disc dislocation, chondrolysis, heterotopic bone formation, and necrosis of the articular tubercle following repeated, high doses of intra-articular corticosteroid (triamcinolone). Surgery was necessary to correct the resulting joint degeneration and limited range of motion (204). In contrast, hyaluronic acid itself does not cause bone necrosis. This complication has nevertheless been reported following hyaluronic acid injections to the TMJ, and is attributed to bone trauma occurring during the intra-articular injection procedure (203, 206). This section reviews the efficacy of corticosteroid and hyaluronic acid injections for TMJ disorders, and highlights the unmet needs in existing intra-articular drug delivery strategies.

### 7.2.1. Corticosteroids

Reports of intra-articular corticosteroid injections to the TMJ date back to over 50 years ago (207). Numerous corticosteroid formulations are available for intra-articular injection, ranging from solutions of more soluble agents to suspensions of triamcinolone hexacetonide and other relatively insoluble steroids. Although the efficacy of various corticosteroids is presumed to differ, studies of this topic have been limited (199, 208,
A variety of methods are currently used for intra-articular corticosteroid injection to the TMJ, each with the goal of minimizing the potential for tissue damage.

Intra-articular corticosteroid formulations are often diluted with a local anesthetic prior to injection into the TMJ (18, 210, 211). This method is thought to decrease the risk of soft tissue atrophy and other complications, although evidence supporting this claim is largely anecdotal (208, 209). In a controlled study of adults with TMJ arthritis, a single intra-articular injection of corticosteroid (methylprednisolone) diluted with lidocaine significantly reduced joint pain and other symptoms for 4-6 weeks (211). The pharmacologic effect of intra-articular methylprednisolone lasts 3-4 weeks, so these findings were consistent with the expected timeline of corticosteroid effect. No adverse events were reported (211).

The most common treatment strategy is either a single injection (18, 211-214) or a series of two injections spaced 14 days apart (215-219), although the best method has yet to be determined. Some clinicians have suggested that a single corticosteroid injection is beneficial for patients with severe TMJ pain, while further injections do not provide added pain relief, and may increase the risk of joint degeneration and other complications (204, 212). In a recent study of patients with TMJ arthritis, there was a significant improvement in TMJ mobility and symptoms, particularly pain and difficulty eating, following the first corticosteroid (triamcinolone) injection. No significant improvement was seen in patients given further injections, suggesting that a single injection is of greatest utility. One patient even developed subcutaneous atrophy after receiving a total of 5 injections to a single TMJ and required surgery (220). This isolated case underscores the need for new methods of intra-articular drug delivery, particularly sustained release
formulations, which would eliminate the need for numerous injections, each of which induces further damage to already degenerating TMJ tissue.

Several decades ago, Toller (212) suggested that intra-articular corticosteroid injections were only useful in adult patients with TMJ disorders; a single intra-articular injection resulted in resolution of TMJ pain and other symptoms in 62% of adult patients, compared to only 17% of pediatric patients (212). However, the efficacy may vary depending on the specific cause of TMJ degeneration. In recent studies of juvenile idiopathic arthritis, intra-articular corticosteroid (triamcinolone) injections improved or even completely eliminated TMJ pain in 77-88% of children for several months (213, 214, 220). Despite the young age of the patients, adverse events (e.g. facial swelling, asymptomatic intra-articular calcifications, and subcutaneous atrophy) were rare in these studies. This likely reflects the cautious injection methods used, which included general anesthesia and costly radiographic needle guidance (213, 214, 220).

As with any clinical technique, the accuracy of placement of intra-articular injections depends upon the experience of the medical practitioner. An estimated one-third to one-half of all steroid and hyaluronic acid injections are inaccurately placed, although the impact of this extra-articular placement on therapeutic efficacy and clinical outcome remains unclear (221, 222). Extra-articular injection of corticosteroids may be more difficult to detect based on patient symptoms because steroid formulations are less viscous than hyaluronic acid preparations, making it more likely that an extra-articular injection of corticosteroid would be painless (219). Needle placement can be confirmed with radiographic or arthroscopic techniques, but these techniques are inconsistently used because they are not considered cost-effective in many cases. Imaging guidance during
intra-articular TMJ injection can double the cost of the procedure (220). Novel methods of long-term intra-articular drug delivery would decrease the number of injections needed and thus decrease the financial burden associated with needle-guidance techniques and other precautions.

Radiographic imaging is a common method of monitoring the progression of TMJ disorders. Long-term follow-up studies of patients receiving intra-articular corticosteroid injections indicate sustained improvement in TMJ pain and, in some cases, radiographic regression of joint disease after 8-12 years (223, 224). However, TMJ degeneration continues to worsen in many cases (216, 220). Patients with severe damage may be less responsive to intra-articular corticosteroids (210), and require multiple injections to treat persistent, severe TMJ symptoms (214, 220). New methods of intra-articular drug delivery will enable tighter control over the release profile of these agents.

7.2.2. Hyaluronic Acid

Intra-articular hyaluronic acid, also called hyaluronan or sodium hyaluronate, has been used for nearly two decades to treat TMJ disorders (206, 225, 226). Hyaluronic acid (HA) is a normal product of joint tissues that is continuously released into the synovial fluid, where it serves as a lubricant, anti-inflammatory, and pain-reliever. Findings of reduced HA molecular weight and concentration in arthritic joints inspired the development of injectable preparations to restore the properties of synovial fluid, so-called viscosupplementation (227). In TMJ disorders, intra-articular HA is used in two different treatment strategies. It is administered alone for viscosupplementation, and is also used as an adjunct to arthrocentesis, a surgical procedure where the joint is flushed to
remove inflammatory mediators (228). The goal of both methods is to relieve TMJ symptoms and reduce inflammation.

The most common viscosupplementation strategy is a series of either 2 intra-articular HA injections spaced 7-14 days apart (215-219, 229-231), or 5 injections each 7 days apart (203, 232-234). In comparison to corticosteroids, a greater number of HA injections are typically administered because HA has fewer potential complications (218). However, a randomized, double-blind, placebo-controlled trial concluded that even a single HA injection has a beneficial effect; patients with TMJ disc displacement had sustained, significant improvement in symptoms and joint mobility for 6 months (206). Intra-articular HA injections not only reduce pain and improve TMJ function, but also reduce the concentration of various inflammatory mediators in the synovial fluid (229). Since the half-life of HA within the joint is very short, some have proposed that this anti-inflammatory function is a more likely mechanism for the long-term effects of viscosupplementation, while others have suggested that short-term improved lubrication may interrupt the cycle of tissue damage and inflammation (230, 231).

A variation on the viscosupplementation method, so-called pumping injection of HA, has shown great promise as a non-surgical therapy for patients with severely limited mouth opening (TMJ closed lock) (225, 232-235). In this technique, a local anesthetic is first injected into the synovial fluid, and the TMJ is then “pumped” by drawing the fluid into and out of the syringe. After pumping several times, most of the synovial fluid is aspirated and replaced with an intra-articular injection of HA (234). The pumping method provides significant improvement in TMJ pain and joint mobility for months to years (232-235). In a study of patients with non-reducing TMJ disc displacement, 82% of
patients continued to be free of pain 2 years after treatment, even though the disc displacement remained uncorrected (234). However, pumping injections significantly increase the risk of further bone degeneration (235), which may be the result of repeated micro-trauma from the syringe tip (203). Novel slow release HA formulations would decrease the number of injections necessary, reducing iatrogenic joint damage.

The multi-functionality of hyaluronic acid, including lubrication, anti-inflammatory, and analgesic properties, led to the use of intra-articular HA as an adjunct to TMJ arthrocentesis. In this minimally invasive surgical technique, two needles are inserted and the joint space is flushed with about 50-300 mL of saline or Ringer’s lactate, an electrolyte solution. In the combined strategy, HA is injected into the TMJ after arthrocentesis is completed (236, 237). As in viscosupplementation, a common regimen is a series of 5 treatments at weekly intervals (237-239). Although an early trial comparing the combined strategy with traditional TMJ arthrocentesis showed no difference on patient outcome (226), a recent randomized trial indicated the combination therapy was more effective in reducing TMJ pain and improving function for 2 years (236). In another study, 70% of patients reported complete resolution of pain 6 months after combined treatment (237).

A recent systematic review and meta-analysis of all randomized, controlled trials of intra-articular HA injections for TMJ disorders concluded that existing evidence is insufficient to either support or refute the benefit of HA injections (240). Meta-analysis of viscosupplementation studies found no statistically significant short-term improvement compared to placebo (saline) injections, although there was some evidence of long-term benefit. Analysis of HA as an adjunct to arthrocentesis was inconclusive due to a lack of
randomized, controlled trials on this topic (240). Although further validation is needed, preliminary studies of HA are promising, as emphasized by studies directly comparing this treatment to intra-articular corticosteroids.

7.2.3. Comparison

Several randomized comparisons of intra-articular hyaluronic acid (sodium hyaluronate) and corticosteroid (betamethasone) TMJ injections have shown no significant difference between the two treatments (216-218). A meta-analysis of studies from 1985-2002 found no statistically significant short- or long-term benefit of intra-articular HA over corticosteroid injections on improving TMJ disorder symptoms (240). However, in a recent randomized, blinded study, hyaluronic acid injections reduced pain to a greater extent than corticosteroids (219). Both treatments resulted in sustained, significantly reduced TMJ symptoms and improved joint mobility for 1-2 years (218). Such significant positive effects on TMJ range of motion were not seen with placebo (saline) injections (215). Radiographic imaging indicated that equal proportions of each treatment group had stable, improved, and worsened joint degeneration (216), and an equal number of transient side effects were reported for both treatments (219). Mixed reports of the efficacy of intra-articular injections may be a reflection of the heterogeneous nature and etiology of TMJ disorders. Further controlled studies are needed to determine the precise impact of intra-articular injections.

7.2.4. Limitations of Current Intra-Articular Injections

Current methods of intra-articular drug delivery often require frequent injections that have a high financial burden, impact patient quality of life, and also increase the risk
of complications. Immediately following each intra-articular injection, patient joint activity (e.g. chewing, talking, etc.) is restricted so as to minimize the risk of either joint overload or tissue reaction resulting in increased drug clearance (12, 199, 230). Although reports of adverse events, particularly following HA injection, are rare, it is unclear whether this reflects reality. Many older studies focused on positive treatment effects, with inconsistent reporting of adverse events (219, 239). Slow-release intra-articular medications have the potential to eliminate many of these concerns. An increased duration of drug release would decrease the number of injections necessary, reducing both the risk of iatrogenic injury and limitations on patient activity.

7.3. Animal Models for Investigating the Efficacy of Intra-Articular Injections

Animal models are a useful tool for understanding the pathophysiological mechanisms underlying TMJ disorders, and for evaluating the efficacy of intra-articular injections. A variety of animal models have been used to evaluate various aspects of drug delivery to the TMJ, including adverse effects of existing intra-articular formulations and the efficacy of emerging treatments.

Small animal models, such as rats and rabbits, have been used to study the adverse effects of currently used intra-articular corticosteroid and hyaluronic acid formulations, given the concerns that complications are under-reported. Rabbit models have been used to study the effect of both corticosteroid and HA injections on TMJ inflammation (241, 242). A recent study comparing arthrocentesis, viscosupplementation, and combination therapy found that arthrocentesis did not prevent TMJ degeneration in
rabbits, but did have a synergistic effect when used in combination with HA injections (242). Recent studies using rodent models found that both corticosteroid and hyaluronic acid injections caused soft tissue inflammation (243, 244). Several corticosteroid preparations induced soft tissue atrophy and necrosis in a rat model (244). Although intra-articular corticosteroid (dexamethasone) injections significantly reduced TMJ inflammation in another rat model, increased osteoclast activity was noted on histological analysis of the mandibular condyle, raising concerns that intra-articular injections may increase the rate of bone resorption (245). However, the implications for the treatment of human patients are unclear because susceptibility to post-injection joint degeneration varies among species, and may even vary by individual. Repeated intra-articular corticosteroid injections in a primate model at exceedingly high dosages had few adverse effects, in contrast to the severe post-injection joint degeneration seen in a rabbit model (246).

Rodent models are commonly used in studies of TMJ damage and transmission of pain signals to the brain (nociception). Rat models of TMJ inflammation have been developed using a variety of methods ranging from repeated, manual, forced mouth opening to intra-articular injection of complete Freund’s adjuvant, a pro-inflammatory agent (27, 245). Inspired by painful, impaired eating in human patients, computerized meal pattern analysis has been developed as a non-invasive method of quantifying TMJ pain in rat models (24-27). Rats are housed in individual cages equipped with photobeam computer-activated pellet feeders. When a rat consumes a food pellet from the device, an infrared beam at the base of the feeding tray is no longer blocked. The beam is detected, and a signal is sent to a computer, which records the date and time and dispenses a new
food pellet; over time, this generates a record of the animal's feeding behavior (24). Longer meal duration has been verified as a specific marker of TMJ inflammation and pain (25, 26). Meal pattern analysis has been used to study the efficacy of several oral and injectable pharmacologic agents (24-27), and is a promising non-invasive tool for evaluating emerging intra-articular drug delivery systems.

Larger animals such as sheep, pigs, or primates may be preferable if intra-articular injection of drug delivery systems is not feasible in a small animal model. Given the prohibitively high cost of primate studies, pigs are currently considered the ideal choice for biomechanical and tissue engineering studies, since their TMJ anatomy is quite similar to that of humans (197, 247, 248). A recent study in a porcine model of TMJ degeneration indicated that HA viscosupplementation decreases friction on joint cartilage by 50% (249). In an ovine (sheep) model, a series of 5 intra-articular HA injections slowed disease progression more than placebo (saline) and significantly reduced the extent of TMJ damage for 3 months (250, 251). Together with less costly rodent and rabbit models, these large animal models will be useful tools for validating the efficacy of emerging intra-articular drug delivery systems.

7.4. Emerging Intra-Articular Drug Delivery Systems

Current methods of intra-articular drug delivery are complicated by rapid degradation and clearance of injected pharmacologic agents, so that frequent injections and high concentrations are necessary. Microcarrier-based drug delivery systems, including hydrogels, polymeric microparticles, and liposomes, are well-established as methods for sustained release in extra-articular applications. Over the past two decades,
several reports have described the use of controlled release systems for intra-articular applications, but most of these strategies have been evaluated in animal models of knee osteoarthritis and other knee joint disorders [see recent reviews (199, 252)]. This section discusses microparticles, liposomes, and other emerging drug delivery systems that promise to greatly improve intra-articular drug delivery to the TMJ.

Intra-articular hyaluronic acid formulations consist of colloidal dispersions of HA in aqueous solution, which form hydrogel structures (199). In the United States, these agents are the only microcarrier-based intra-articular formulations available for clinical use in any joint. However, as previously stated, intra-articular HA injections are not yet FDA-approved for use in the TMJ (12, 201). Nevertheless, recent progress in the field of injectable biomaterials shows great promise for the development of superior intra-articular therapies. Novel HA-based materials have the potential to improve viscosupplementation and joint regeneration strategies (253, 254). In a recent study, intra-articular delivery of basic fibroblast growth factor using an HA vehicle significantly improved osteochondral repair in the knee joint of a rabbit model (255). Injectable, biodegradable hydrogels have shown promise for delivery of cells and growth factors promoting cartilage regeneration (256-258). Adaptations of these drug delivery systems have the potential to enhance TMJ drug delivery and tissue engineering strategies.

Polymeric nano- and microparticles made from a variety of natural and synthetic materials, including albumin, gelatin, and poly(lactic-co-glycolic acid) (PLGA) are being investigated for intra-articular sustained release applications [see recent reviews (199, 252)], as summarized in Table 7.1.
Table 7.1:  
Particulate intra-articular drug delivery systems that have been studied in vivo

<table>
<thead>
<tr>
<th>Delivery System</th>
<th>Drugs</th>
<th>Animal Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin microparticles</td>
<td>Diclofenac sodium (NSAID)</td>
<td>Rabbit knee</td>
<td>(259)</td>
</tr>
<tr>
<td>Chitosan microparticles</td>
<td>Celecoxib (NSAID), Paclitaxel</td>
<td>Rat knee, Rabbit knee</td>
<td>(260, 261)</td>
</tr>
<tr>
<td>Gelatin microparticles</td>
<td>b-FGF</td>
<td>Rabbit knee</td>
<td>(255, 262)</td>
</tr>
<tr>
<td>Gelatin / chondroitin-6 sulfate microparticles</td>
<td>Albumin, Catalase</td>
<td>Mouse knee</td>
<td>(263)</td>
</tr>
<tr>
<td>PCL microparticles</td>
<td>Paclitaxel</td>
<td>Rabbit knee</td>
<td>(261)</td>
</tr>
<tr>
<td>PLGA microparticles</td>
<td>Paclitaxel, Diclofenac sodium (NSAID)</td>
<td>Rabbit knee, Rat knee</td>
<td>(261, 264, 265)</td>
</tr>
<tr>
<td>PLGA nanoparticles</td>
<td>Betamethasone</td>
<td>Rat knee, Rabbit knee</td>
<td>(264, 266)</td>
</tr>
<tr>
<td>PLLA microparticles</td>
<td>Paclitaxel</td>
<td>Rabbit knee</td>
<td>(261)</td>
</tr>
<tr>
<td>Polyphosphazene-based nanoparticles</td>
<td>Indomethacin (NSAID)</td>
<td>Rat ankle</td>
<td>(267, 268)</td>
</tr>
<tr>
<td>PPS nanoparticles</td>
<td>Oligopeptide</td>
<td>Mouse knee</td>
<td>(269)</td>
</tr>
</tbody>
</table>

In one study, rat knee joints injected with microencapsulated versus aqueous (free drug solution) formulations had a 10-fold difference in NSAID concentration after 24 hours (260). Reports of joint inflammation in vivo following intra-articular injections of
gelatin and chitosan microparticles have caused some concern. Although the cause remains unclear, it has been suggested that these reactions may reflect impurities in the commercially available biomaterials used to create these microparticles, and not an intrinsic proinflammatory property of gelatin and chitosan (261, 263). For instance, a recent study in a rabbit model indicated that intra-articular delivery of basic fibroblast growth factor via gelatin microparticles improved knee joint swelling, proteoglycan expression, and histological appearance of arthritic tissue (262). At the same time, a variety of synthetic carriers, including poly(caprolactone) (PCL)-, poly(L-lactic acid) (PLLA)-, PLGA-, poly(propylene sulphide) (PPS)-, and polyphosphazene-based nano- and microparticles, have also shown promise for intra-articular applications (261, 265, 267-269) (see Table 7.1). The ideal material remains to be elucidated. The optimal size range for intra-articular drug delivery systems is also debated, with some studies reporting that nanoparticles are superior (264, 266), while others support the use of microparticles (259, 261). These and other design parameters will need to be optimized in the development of intra-articular methods of sustained release for the TMJ.

A liposomal corticosteroid formulation containing dexamethasone-21-palmitate (Lipotalon®) available in Germany is the only intra-articular liposomal product used in human patients (199). As with microcarriers, many preclinical studies have investigated the efficacy of intra-articular liposomal drug delivery systems [see recent review (252)]. In an effort to eliminate joint injections altogether, intravenous administration of these preparations has also been evaluated. However, in a recent study, intravenous administration of a liposomal formulations of an anti-inflammatory agent failed to treat arthritic symptoms in a rat model (270). In contrast, a single intra-articular injection
suppressed rat knee joint inflammation for 3 weeks. Large multilamellar (micro-scale) liposomes were more effective than small unilamellar (nano-scale) liposomes (271). These novel controlled release systems may provide a better solution for intra-articular administration of various compounds to the TMJ.

One particularly promising application is intra-articular delivery of small interfering ribonucleic acids (siRNAs), endogenous molecules that “silence” specific genes by binding to messenger RNA and blocking protein translation (272, 273). Although synthetic siRNA formulations that suppress the vascular endothelial growth factor pathway are already in clinical trials for treatment of pathologic neovascularization within the eye (272, 273), development of joint therapeutics remains in the preclinical stage. A major challenge is the lack of a method for intra-articular sustained release (274, 275). In two recent studies in rodent models of knee arthritis, intra-articular delivery of siRNA targeting tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, significantly reduced inflammation and exerted a chondroprotective effect for several weeks (276, 277). A similar beneficial effect on murine knee joint inflammation was reported with systemic administration of anti-TNF-α siRNA complexed with cationic liposomes (278). However, all of these strategies required multiple injections to achieve these positive effects. Long-term treatment of joint disease via siRNA will require novel sustained release strategies. Various groups are already investigating methods of intra-articular siRNA delivery in animal models of TMJ inflammation, and preliminary findings are promising.

Emerging methods of intra-articular drug delivery have the potential to overcome many of the limitations of existing intra-articular formulations. To our knowledge, none
of the strategies discussed in this section has yet been evaluated in a TMJ model. Novel systems for sustained release will increase the residence time of medications within the joint, reducing the need for repeated intra-articular injections and thus minimizing iatrogenic damage. Tighter control over release kinetics would reduce the required medication dosage and decrease the risk of ectopic effects.

7.5. Importance of Drug Delivery for TMJ Tissue Engineering

Advances in the field of drug delivery will improve pain management and also aid progress in the emerging field of TMJ regeneration. The standard treatment for severe TMJ degeneration is currently surgical joint replacement (279). Research in TMJ repair and regeneration has proceeded at a cautious pace following the experience with early alloplastic TMJ implants, whose tendency for premature failure triggered immune reactions resulting in catastrophic joint damage (197, 280). Although improved products are currently available for total joint replacement, even these have a limited lifetime. The potential need for serial surgeries to replace worn out prostheses has raised concerns, since numerous clinical studies have indicated that serial TMJ surgeries often result in joint mutilation (15, 279).

Drug delivery systems can be used to either modify the joint environment prior to implantation, or to deliver proregenerative signals in a spatially and temporally controlled fashion, so as to trigger joint healing while avoiding systemic drug release, ectopic effects, and other such complications. The limitations of current therapeutic strategies for TMJ disorders have led to increased interest in tissue engineering strategies, which combine cells, bioactive factors, and implantable scaffolds to trigger joint regeneration
(281). However, TMJ tissue engineering remains a small and emerging field. Research to date has focused on separately engineering the fibrocartilagenous TMJ disc and mandibular condyle, with few preclinical or in vivo studies [see recent reviews of TMJ disc (282, 283) and mandibular condyle tissue engineering (284)].

Since joint tissue, particularly the fibrocartilagenous disc, has very limited healing capacity, TMJ tissue engineering strategies must deliver key growth factors and other molecular signals that induce joint regeneration (285). Development of systems for controlled release of these factors will be critical to the success of TMJ regeneration (286). Tissue engineered products may never work properly unless researchers identify and find a means to control the underlying joint degeneration. The emerging intra-articular drug delivery systems described in this paper have the potential to both treat pain associated with TMJ disorders and deliver growth factors and other signals to control the underlying joint degeneration.

7.6. Concluding Remarks

TMJ disorders are a group of degenerative joint diseases that cause progressive damage to the fibrocartilagenous TMJ disc, mandibular condyle, and synovial tissues. Although there has been significant progress in both drug delivery and joint tissue engineering, challenges still remain, including the need for sustained release preparations and minimally invasive tissue engineering approaches to reduce iatrogenic joint damage. Tissue engineering efforts have separately focused on the mandibular condyle and TMJ disc. However, it seems unlikely that the disc could be implanted alone, so future efforts will need to focus on developing an implantable disc-condyle combination. In addition to
providing a means of sustained release of pain-relieving medications, intra-articular controlled release systems have the potential to play a crucial role in the clinical implementation of tissue engineering strategies for TMJ regeneration.
Chapter 8

Intra-articular Microparticles for Drug Delivery to the TMJ

Abstract: This study describes the in vivo biocompatibility of intra-articular poly(DL-lactic-co-glycolic acid) (PLGA) microparticle (MP) formulations in the rat temporomandibular joint (TMJ). To our knowledge, this is the first intra-articular microparticle-based drug delivery system for the TMJ. The impact of PLGA MP concentration on rat TMJ function was quantified via computerized meal pattern analysis; in this non-invasive technique, previously validated markers of TMJ pain (specifically, meal duration and food intake) were recorded using computer-monitored pellet feeders. Bilateral intra-articular injection of 15, 30, or 50 mg/mL PLGA MPs had no impact on

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meal duration or food intake over six days, compared to controls that did not receive injections. Histological analysis showed that the MPs were retained within the synovial lining. These findings indicate that the PLGA MPs described herein are biocompatible and suitable for intra-articular delivery to the rat TMJ, a finding that has significant implications for the improvement of TMJ therapeutics.

8.1. Introduction

Temporomandibular joint (TMJ) disorders are a heterogeneous group of diseases that cause pain (12), resulting in an estimated $4 billion per year in treatment costs in the United States. Unlike other joint diseases, TMJ disorders have a higher incidence in adolescents and young adults (194). Effective pain reduction and restoration of TMJ function remain unmet challenges (12, 13). In cases of TMJ degeneration, intra-articular injections of corticosteroids and hyaluronic acid are used to treat pain. However, these intra-articular formulations are complicated by rapid clearance of injected agents, so that frequent injections are needed, increasing the risk of iatrogenic injury (12, 13). There is a need for sustained release formulations to effectively alleviate TMJ pain.

Over the past two decades, microcarrier-based drug delivery systems, particularly polymeric microparticles, have been evaluated as novel strategies for intra-articular controlled release in animal models of knee joint disorders [recent reviews, (13, 287)]. Poly(DL-lactic-co-glycolic acid) (PLGA) microparticles (MPs) have been successfully applied for intra-articular controlled release in the knee joints of rabbits, rats, and mice (261, 264, 265, 288). However, to our knowledge, no intra-articular microparticle-based system has yet been evaluated in the TMJ (13).
In this study, we used an established double emulsion solvent extraction technique to generate PLGA MPs previously shown to be capable of controlled release of bioactive molecules including proteins (289-291). We evaluated the \textit{in vivo} biocompatibility of these PLGA MPs following bilateral intra-articular injection into rat TMJs using a non-invasive method, computerized meal pattern analysis. This technique, whose development was inspired by the painful, impaired eating in human patients with TMJ disorders, has been used to evaluate the effect of various analgesic agents on TMJ pain (22, 24-27). Increased meal duration has been shown to be a specific marker of TMJ pain (26), and reduced food intake is often associated with this trend, e.g., due to exacerbation of pain by chewing (22).

This study addresses the following questions: (i) Are PLGA MPs biocompatible \textit{in vivo} following intra-articular injection into the TMJ, and (ii) what is the impact of intra-articular PLGA MP concentration on TMJ function? We hypothesized that all concentrations of intra-articular PLGA MPs would be well tolerated in healthy rat TMJs. Our ultimate goal was to determine the suitability of PLGA MPs for intra-articular sustained release in the rat TMJ.

\section*{8.2. Materials and Methods}

\subsection*{8.2.1. PLGA microparticle preparation and characterization}

PLGA microparticles consisting 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) were synthesized using a previously described double emulsion solvent extraction technique.
For fluorescent MPs, 27 µL of Vybrant DiI (Molecular Probes, Carlsbad, CA) were added prior to generating the first emulsion. To prevent quenching of the fluorescent dye, all vials were wrapped with aluminum foil and indirect lighting was used. The number average molecular weight (Mn) of the PLGA 50:50 was 42500 ± 1600 Da and its polydispersity index was 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). MP diameter was determined using a Multisizer3 Coulter Counter (Beckman Coulter, Fullerton, CA). MP morphology was observed via scanning electron microscopy (SEM; FEI Quanta 400 Environmental, Hillsboro, OR) at 15 kV accelerating voltage, after coating the MPs with a thin layer of gold.

8.2.2. *In vivo* microparticle localization

All rat studies were approved by the Institutional Animal Care and Use Committee and adhered to the National Institutes of Health guidelines. Before assessing the impact of intra-articular MPs on TMJ function, a pilot study was conducted to localize the MPs immediately after injection. Pre-weighed fluorescent PLGA MPs were sterilized via exposure to ethylene oxide gas for 14h, aired out for 24h, and then suspended at 50 mg/mL in Microfil (Flowtech, Carver, MA), which was prepared in a 4:5 Microfil:diluent volume ratio with 5% v/v curing agent. Two adult male Sprague-Dawley rats (Harlan Industries, Houston, TX) (250-300g) were anesthetized with isoflurane (5% v/v in oxygen) and received bilateral 50 µL TMJ injections into the superior joint space according as previously described (22, 26, 27). All rats were euthanized immediately afterward with a carbon dioxide overdose. The Microfil was then allowed to cure for 3-4
h, so that it would encase the fluorescent dye-loaded PLGA MPs. Each TMJ was then removed *en bloc*, fixed in 10% v/v formalin for two days, and embedded in acrylic. Tissue sections (500 μm) were not stained to prevent quenching of the fluorescent dye. Phase contrast and fluorescence (excitation: 515-575 nm; emission: 560-680 nm) images were captured using a Nikon Eclipse 80i microscope (Melville, NY) with a Photometrics CoolSNAP K4 camera (Tucson, AZ) and Metamorph software (Sunnyvale, CA).

### 8.2.3. Meal pattern analysis

35 healthy adult male Sprague-Dawley rats (Harlan Industries) (250-300g) were housed in previously described computer-activated pellet feeder cages (22, 26). After 2 days to acclimate, 32 rats received bilateral 50 μL TMJ injections as described above. The injected material consisted of 15, 30, or 50 mg sterile PLGA MPs ($n = 8$ rats/group) per mL of sterile 10% v/v Tween 80 (Sigma, St. Louis, MO) in normal (0.9% w/v) saline. Tween 80 was necessary to prevent MP aggregation, which enabled aspiration and injection with a fine bore (25 gauge) needle that minimized iatrogenic TMJ damage. The remaining rats received carrier solution only ($n = 8$) or ($n = 3$) had no injections.

Meal duration and food intake were recorded for 6 days after injection using the computer-activated pellet feeders, as previously described (22, 27). After 6 days, all rats were deeply anesthetized (9 mg ketamine and 1.5 mg Rompun per 100 g body weight) and perfused with 4% w/v paraformaldehyde in PBS (pH 7.0). The TMJs were removed *en bloc* and demineralized using an established microwave-accelerated technique (292). Sagittal cryosections (20 μm) were imaged using a Zeiss Axioplan microscope (Thornwood, NY) with a Diagnostic Instrument Inc., Spot CCD camera (Sterling Heights, MI).
8.2.4. Statistical Analysis

Meal pattern data are reported as mean ± standard error of the mean, and represent a percentage of the average pre-injection value. Data were analyzed using two-way analysis of variance ($p < 0.05$), followed by Bonferroni post-hoc analysis ($p < 0.05$).

8.3. Results

8.3.1. PLGA MP morphology

The MPs had a diameter of $22 \pm 7 \mu m$ (mean ± standard deviation; $n = 1500$ MPs). SEM imaging (Figure 8.1d) confirmed that they had spherical morphology and size consistent with Coulter counter measurements.

8.3.2. In vivo microparticle localization

Injections of the fluorescent-dye loaded PLGA MPs were successfully made into the superior joint space of the TMJ. The hardened Microfil (Figure 8.1a, solid outline) held the MPs in place within the superior joint space (Figure 8.1a, dashed outline) during tissue processing. By fluorescence microscopy, the dye-loaded microparticles were visible within the pores of the Microfil (Figure 8.1b), and individual MPs could be resolved at higher magnification (Figure 8.1c).
Figure 8.1: In vivo PLGA MP localization

Phase contrast and fluorescence microscopy images indicated successful injection of fluorescent-dye-loaded PLGA microparticles (MPs) into the superior joint space. In this representative unstained histological section from a rat euthanized immediately after injection (2.5× magnification), (a) the Microfil (solid outline) containing the PLGA MPs is visible within the superior joint space (dashed outline) via phase contrast imaging. A higher magnification (4×) fluorescent image of this same section confirms that (b) the fluorescent-dye-loaded MPs are contained within the Microfil (arrow points to an area with MPs), with (c) individual MPs (white arrows) visible within the Microfil at 40× magnification. A scanning electron micrograph (d) of the spherical PLGA microparticles is included for comparison.
8.3.3. Impact of intra-articular microparticles on meal patterns

Intra-articular injection of PLGA MPs did not significantly increase \((p > 0.05)\) meal duration (Figure 8.2a) or decrease \((p > 0.05)\) food intake (Figure 8.2b) compared to baseline. These findings are particularly striking when compared to the “Inflamed TMJ *” values (Figure 8.2a,b), which represent the previously reported response following bilateral injection of complete Freund’s adjuvant (CFA). The 0 mg/mL PLGA control differed significantly \((p < 0.05)\) from the 15 and 50 mg/mL PLGA groups in terms of meal duration (Fig. 2a), and from the 30 mg/mL PLGA group in terms of food intake (Figure 8.2b). However, none of the groups (Figure 8.2a,b) differed significantly from the “No injection” control \((p > 0.05)\).
Figure 8.2a – figure continued on next page
Figure 8.2: Impact of empty PLGA MPs on meal parameters
Intra-articular microparticles did not induce TMJ pain, based on (a) meal duration [previous page], and (b) food intake measurements [this page] for two days prior and six days following intra-articular injection of 15, 30, or 50 mg/mL PLGA microparticle formulations (abbreviated “PLGA”). A dashed line indicates the time of injection. The upper panels of both (a) and (b) depict the two control groups, consisting of rats that did
not receive any injections ("No injection"), and rats where only the carrier fluid was injected ("0 mg/mL PLGA"). (a) Meal duration differed significantly between the 0 mg/mL PLGA group and both the 15 mg/mL PLGA and 50 mg/mL PLGA groups (\( p < 0.05 \); indicated by #). However, none of the groups differed from the "No injection" negative control (\( p > 0.05 \)). Meal duration data from a previous study where rat TMJ inflammation was induced via bilateral injection of a pro-inflammatory agent (15 \( \mu \)g CFA/joint; "Inflamed TMJ *" group) are included in (a) for comparison. In that study, a significant increase (\( p < 0.05 \)) in meal duration was observed compared to the baseline pre-injection value (27). None of the groups in this study showed a significant increase in meal duration compared to baseline (\( p > 0.05 \)). (b) Food intake differed significantly (\( p < 0.05 \)) between the 30 mg/mL PLGA group and both the 15 mg/mL PLGA experimental group and the 0 mg/mL PLGA control group. However, none of the groups differed from the "No injection" negative control (\( p > 0.05 \)). Food intake results from a previous study where rat TMJ inflammation was induced via bilateral injection of a pro-inflammatory agent (300 \( \mu \)g CFA/joint; "Inflamed TMJ *" group) are included in (b) for comparison. In that study, a significant decrease (\( p < 0.05 \)) in food intake was observed compared to the baseline pre-injection value (22). None of the groups in the present study showed a significant decrease in food intake compared to the baseline values (\( p > 0.05 \)). Values for each group are expressed as a percentage of the average pre-injection value. Data points represent the mean ± standard error of the mean for \( n = 8 \) rats (or \( n = 3 \) rats in the "No injection" group). Error bars are included for all groups, though they are too small to resolve in some cases. Groups that differ significantly (\( p < 0.05 \)) from "0 mg/mL PLGA" are indicated by #.

### 8.3.4. In vivo biocompatibility of intra-articular microparticles

Injection of the carrier solution induced a mild inflammatory infiltrate, and no additional tissue reaction was observed in the 15, 30, or 50 mg/mL PLGA groups. The MPs were embedded within the posterior synovial tissue (arrows, Figure 8.3) and were surrounded by leukocytes (hematoxylin-stained blue nuclei, Figure 8.3b,c) that often had the nuclear morphology of macrophages.
Figure 8.3: Stained histological sections of TMJ tissue with PLGA MPs

Representative images from the 30 mg/mL PLGA group indicating (a) the location of the PLGA MPs (arrows), which were primarily embedded within the synovial tissue lining the posterior surface of the superior joint space, adjacent to the TMJ vasculature. The specific MPs identified in (a) are shown at higher (40×) resolution in (b) and (c), again indicated by arrows. The MPs were surrounded by inflammatory cells (blue nuclei), but were too large to be engulfed by these cells. All histological sections shown were stained with hematoxylin and eosin. The 50 μm error bar in (c) also applies to (b). The original magnification of (a) was 2.5×.
8.4. Discussion

In this study, we report the development and *in vivo* biocompatibility of a PLGA MP formulation for intra-articular drug delivery in the rat TMJ. Although microparticle-based controlled release systems exist for various applications, to our knowledge, this is the first such system for intra-articular delivery in the TMJ. Due to the lack of previous data regarding the TMJ tissue response to such systems (13), this study utilized blank PLGA MPs injected into healthy rat TMJs. Another reason for this cautious approach was the terrible history of early alloplastic (Proplast-Teflon) TMJ implants, whose susceptibility to mechanical failure gave rise to Teflon microparticles that triggered severe immune reactions and catastrophic TMJ damage (15).

PLGA MPs were selected based on their successful intra-articular application in rodent and rabbit knees (261, 264, 265, 288). Although knee joint results cannot be directly applied to the TMJ due to significant differences amongst the two joints (247), we hypothesized that the tissue response would be similar in both cases. MPs (22 ± 7 μm diameter; Figure 8.1d) were selected over smaller particles based on a previous report that similar PLGA MPs (27 ± 9 μm diameter) were retained in rat knee joint synovial tissue, while PLGA nanoparticles (265 ± 15 nm) were not. The nanoparticles were phagocytosed by macrophages responding to the injury associated with injection. Within a week, these macrophages migrated away from the synovial lining, taking the nanoparticles along (264). We chose MPs in order to maximize retention within the TMJ. At the same time, PLGA MP diameter was kept small (~20 μm) to facilitate delivery with the thin needle necessary to minimize iatrogenic injury.
The ability to deliver a high concentration of microparticles is important for establishing a depot capable of delivering a sufficient drug dose. The highest dose of MPs (50 mg/mL) was the maximum achievable suspension that could be reproducibly aspirated and delivered via the 25 gauge needle necessary for rat TMJ injections. This concentration exceeds values of intra-articular PLGA MPs delivered in saline to rodent knees (264, 288), and is similar to PLGA MP doses delivered to much larger joints, e.g., rabbit knees (261). The high PLGA MP concentrations achieved in our study may be due to the addition of Tween 80, which reduced MP aggregation and facilitated intra-articular delivery. This agent is already approved by the United States Food and Drug Administration (FDA) as an inactive component of intra-articular injection formulations (293). Injection of 50 μL of the 50 mg/mL formulation resulted in intra-articular delivery of 2.5 mg of PLGA MPs per TMJ. This amount, together with drug loading efficiency, is an important design parameter for future studies with drug-loaded MPs, as it will determine the maximum achievable drug dose.

Injection of 15, 30, or 50 mg/mL PLGA MPs caused no additional tissue reaction after one week, compared to the carrier solution alone. The mild inflammatory infiltrate observed (Figure 8.3) is consistent with the rat and rabbit knee response one week after injection of 40-75 mg/mL PLGA MPs (261, 264). Future studies will probe the long-term biocompatibility of intra-articular PLGA MPs in the TMJ. The localization of the MPs within the adipocyte-rich posterior TMJ synovial tissue (Figure 8.3) is consistent with reports for PLGA MPs in rodent knees (264, 288) and has been attributed to the hydrophobicity of the polymer (294). The biocompatibility of the MPs is underscored when compared to rat TMJ response to CFA injections, which resulted in severe
inflammation that was worst in the posterior synovial tissue adjacent to the TMJ vasculature (22), the same anatomical region where the PLGA MPs were localized in the present study. PLGA MPs within the TMJ synovium were surrounded by inflammatory cells (blue nuclei in Figure 8.3b,c), as reported for rodent and rabbit knees, where PLGA MPs that were too large to be phagocytosed were instead enveloped by macrophages (264, 287, 288). Future studies will build upon these promising findings, to determine whether PLGA MPs are also biocompatible in the diseased TMJ.

Computerized meal pattern analysis has been extensively validated in rat TMJ disorder models (22, 24-27). The use of this non-invasive method of quantifying TMJ pain enabled repeated measurements of each rat. Remarkably, the PLGA MP formulations had essentially no effect on meal duration (Figure 8.2a) and food intake (Figure 8.2b), both markers of TMJ pain. Although some groups differed from the 0 mg/mL PLGA (carrier solution only) control, these statistical differences are unlikely to be scientifically significant because the magnitude of the difference is extremely small when compared to the changes induced by inflammatory agents ("Inflamed TMJ *" group in Figure 8.2a,b). Moreover, none of the groups differed from the "No injection" negative control ($p > 0.05$).

In this work, we report the in vivo biocompatibility of PLGA MPs for intra-articular sustained release in the rat TMJ. We found that bilateral intra-articular injection of 15, 30, or 50 mg/mL PLGA MPs had no impact on rat TMJ function over six days, as determined by histology and computerized meal pattern analysis. This highlights the exciting potential of these formulations to serve as the first intra-articular controlled release system for the TMJ, and thus greatly improve TMJ therapeutics.
Chapter 9

Controlled Release of Anti-Inflammatory siRNA from Biodegradable Polymeric Microparticles Intended for Intra-articular Delivery to the Temporomandibular Joint

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ABSTRACT

Purpose: As the next step in the development of an intra-articular controlled release system to treat painful temporomandibular joint (TMJ) inflammation, we developed several biodegradable poly(DL-lactic-co-glycolic acid) (PLGA)-based microparticle (MP) formulations encapsulating a model anti-inflammatory small interfering RNA (siRNA) together with branched poly(ethylenimine) (PEI) as a transfecting agent. The effect of siRNA loading and N:P ratio on the release kinetics of siRNA-PEI polyplexes was determined, and the size and N:P ratio of the polyplexes released over time was characterized.

Methods: Polyplex-loaded PLGA MPs were prepared using an established double emulsion technique. Increasing the pH of the release samples enabled siRNA-PEI dissociation and subsequent measurement of the release of each component over 28 days. Polyplex diameter was measured for all release samples and compared to freshly prepared siRNA-PEI under simulated physiologic conditions.

Results: Systematic variation of siRNA loading and N:P ratio resulted in distinct siRNA and PEI release profiles. Polyplex diameter remained constant despite large variations in the relative amounts of siRNA and PEI. Excess PEI was sequestered through complexation with 500-1000 nm diameter PLGA MP-derived particles, including small MPs and PLGA degradation products.

Conclusions: These PLGA MP formulations show exciting potential as the first intra-articular TMJ controlled release system.
9.1. Introduction

Temporomandibular joint (TMJ) disorders are a heterogeneous group of diseases that cause painful, progressive joint degeneration that limits talking, chewing, and other basic activities (12). In severe TMJ degeneration, pain can no longer be treated with oral medication, due to the risk of systemic toxicity, and thus intra-articular injections of corticosteroids or hyaluronic acid are required. However, current intra-articular formulations are complicated by rapid clearance of injected agents, requiring frequent injections that carry a high risk of iatrogenic joint injury. Due to these limitations, effective pain reduction and restoration of TMJ function remain unmet challenges (12, 13).

The goal of this study is to develop a sustained release system for a model anti-inflammatory agent: small interfering RNA (siRNA) targeting the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α). TNF-α was chosen as the drug target because it is highly expressed in the TMJs of patients and animal models with painful inflammation (18, 19). Previous studies in rodent knee joint models have indicated that intra-articular injection of 5-10 μg of anti-TNF-α-siRNA effectively treats inflammation. However, these treatment strategies required multiple intra-articular injections (e.g., once a week for three weeks) and electroporation of the affected joints to induce intracellular uptake of the charged siRNA and achieve long-term treatment of joint inflammation (276, 277). The delivery of anti-TNF-α siRNA to cells results in degradation of mRNA encoding TNF-α, thus silencing gene expression by preventing translation of the mRNA into protein. Gene silencing via siRNA is a promising therapeutic strategy that requires
development of a delivery system that both protects the nucleic acid from degradation and also facilitates its uptake into cells (295, 296).

We have previously reported the in vivo biocompatibility of blank poly(DL-lactic-co-glycolic acid) (PLGA)-based microparticles (MPs) injected intra-articularly in the rat TMJ (16). PLGA MPs have been used for a wide variety of drug delivery applications, and a few recent reports have described the in vitro and in vivo efficacy of PLGA-based MPs (297, 298) and nanoparticles (299-302) in delivering siRNA, typically in conjunction with a transfection agent such as poly(ethylenimine) (PEI). PEI protects siRNA from degradation by ubiquitous extracellular nucleases, and balances the negative charge of siRNA which would otherwise impair cellular uptake (296). The ratio of PEI to siRNA is commonly expressed as the Nitrogen:Phosphate (N:P) ratio, i.e., the ratio of protonatable amine (N) groups to nucleic acid backbone phosphates (P). The objectives of this study were to develop various siRNA-PEI-loaded PLGA MP formulations and then to determine the effect of siRNA loading and N:P ratio on the release kinetics of the siRNA-PEI polyplexes.

9.2. Materials and Methods

9.2.1. Experimental Design

A factorial design was used to evaluate the impact of siRNA loading and N:P ratio on polyplex release from PLGA MPs over 28 days. Two levels of theoretical siRNA loading (~0.07 and ~0.35 μg siRNA / mg PLGA MPs) were combined with two N:P ratios (8:1 and 16:1), resulting in 4 distinct siRNA-PEI-loaded PLGA MP formulations, as summarized in Table 9.1 and Table 9.2. For siRNA release, control groups consisted of
siRNA-only-loaded PLGA MPs with equivalent “high” or “low” siRNA loading (Table 9.1) to that of the polyplex-loaded MPs. For PEI release, control groups consisted of PEI-only-loaded MPs with PEI content equivalent to the lowest and highest PEI theoretical loading values (corresponding to the “low siRNA, 8:1 N:P” and “high siRNA, 16:1 N:P” groups, respectively) (Table 9.2). For polyplex size measurements, blank PLGA MPs were included as a control group to enable differentiation of polyplexes and PLGA MP derivatives, such as degradation products. Freshly prepared polyplexes were included in the study design to elucidate the effect of release from PLGA MPs on siRNA-PEI polyplex diameter.
<table>
<thead>
<tr>
<th>siRNA loading</th>
<th>N:P ratio</th>
<th>PLGA MP yield (%)</th>
<th>Theoretical siRNA loading (μg siRNA / mg PLGA MPs)</th>
<th>Entrapment efficiency (% of theoretical loading)</th>
<th>MP diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low 8:1</td>
<td>88 ± 3</td>
<td>0.062 ± 0.002</td>
<td>61 ± 13</td>
<td>28 ± 4</td>
<td></td>
</tr>
<tr>
<td>No PEI</td>
<td>68 ± 1</td>
<td>0.080 ± 0.001</td>
<td>55 ± 5</td>
<td>27 ± 5</td>
<td></td>
</tr>
<tr>
<td>High 8:1</td>
<td>85 ± 2</td>
<td>0.32 ± 0.01</td>
<td>27 ± 5</td>
<td>32 ± 2</td>
<td></td>
</tr>
<tr>
<td>No PEI</td>
<td>72 ± 4</td>
<td>0.37 ± 0.01</td>
<td>39 ± 4</td>
<td>26 ± 8</td>
<td></td>
</tr>
</tbody>
</table>

a Actual weight of PLGA MPs from each batch as a percentage of the starting amount of PLGA and PEG (248 mg per batch)
b Calculated by dividing the amount of siRNA added during synthesis (13.4 μg for low and 67.0 μg for high loading) by the actual weight of PLGA MPs from that batch (= yield × 248 mg)
c Entrapment efficiency of siRNA calculated from: (amount of siRNA extracted from 15 mg PLGA MPs) / (theoretical siRNA loading) × 100%
d Determined via Coulter Counter analysis (n = 1500 microparticles per batch)
e Differs significantly from all other entrapment efficiencies except that of low siRNA, 8:1 N:P (p < 0.05)
f Values do not differ from each other but differ from all other entrapment efficiencies (p < 0.05)
g Differs significantly from all other entrapment efficiencies (p < 0.05)
9.2.1. Polyplex preparation

The siRNA sequence (sense: 5'-CCCACGUCGUAGCAAACCTT-3'; Silencer® Select siRNA ID no. s128524, Ambion, Austin, TX) was selected to closely match a sequence shown to successfully treat rat knee inflammation in vivo (276). The siRNA was reconstituted at a concentration of 0.1 nmol siRNA/µl in nuclease-free water according to the manufacturer’s instructions. To create polyplexes with a 16:1 N:P ratio, a portion of this siRNA stock solution (10 µl for “low siRNA loading” and 50 µl for “high siRNA loading”) was mixed with an appropriate volume (14.3 µl and 72 µl, respectively) of a 2 mg/ml solution of branched PEI (nominal weight-average molecular weight 25 kDa, Aldrich, Milwaukee, WI) dissolved in phosphate-buffered saline (PBS). The mixture was vortexed for a few seconds, incubated at room temperature for 20 minutes to allow for polyplex formation, and then immediately used to prepare (siRNA-PEI)-loaded PLGA MPs. For polyplexes with an 8:1 N:P ratio, half the volume of PEI solution was used. N:P ratios were calculated as previously described (303). For formulations 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 Biotechnology, Rockford, IL). A single r-PEI batch was used for all experiments described herein. To prevent quenching of the rhodamine, all vials were wrapped with aluminum foil and indirect lighting was used.
Table 9.2: Entrapment efficiency of PEI in the PLGA microparticles

<table>
<thead>
<tr>
<th>siRNA loading</th>
<th>N:P ratio</th>
<th>PLGA MP yield (%)(^a)</th>
<th>Theoretical PEI loading (µg PEI/mg PLGA MPs)(^b)</th>
<th>Entrapment efficiency (% of theoretical loading)(^c)</th>
<th>MP diameter (µm)(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>n/a</td>
<td>72 ± 1</td>
<td>0.08 ± 0.00</td>
<td>45 ± 4(^e)</td>
<td>29 ± 3</td>
</tr>
<tr>
<td></td>
<td>n/a</td>
<td>70 ± 1</td>
<td>0.82 ± 0.01</td>
<td>66 ± 2</td>
<td>29 ± 2</td>
</tr>
<tr>
<td>Low</td>
<td>16:1</td>
<td>69 ± 1</td>
<td>0.17 ± 0.01</td>
<td>73 ± 12</td>
<td>32 ± 4</td>
</tr>
<tr>
<td></td>
<td>8:1</td>
<td>66 ± 1</td>
<td>0.09 ± 0.00</td>
<td>75 ± 12</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>High</td>
<td>16:1</td>
<td>72 ± 2</td>
<td>0.81 ± 0.05</td>
<td>51 ± 6(^e)</td>
<td>29 ± 2</td>
</tr>
<tr>
<td></td>
<td>8:1</td>
<td>69 ± 1</td>
<td>0.42 ± 0.00</td>
<td>72 ± 8</td>
<td>34 ± 2</td>
</tr>
</tbody>
</table>

\(^a\) Actual weight of PLGA MPs from each batch as a percentage of the starting amount of PLGA and PEG (248 mg per batch)

\(^b\) Calculated by dividing the amount of r-PEI added during synthesis (28.6 µg for low siRNA, 16:1 N:P; 14.3 µg for low siRNA, 8:1 N:P; 143 µg for high siRNA, 16:1 N:P; and 71.6 µg for high siRNA, 8:1 N:P) by the actual weight of PLGA MPs from that batch (= yield × 248 mg)

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

\(^d\) Determined via Coulter Counter analysis (n = 1500 microparticles per batch)

\(^e\) Indicates values that do not differ from each other but differ from all other entrapment efficiencies (p < 0.05)
9.2.2. PLGA microparticle preparation and characterization

The PLGA microparticles 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 number-average molecular weight of the PLGA was 42500 ± 1600 Da and its polydispersity index was 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). MPs were synthesized using a previously described double emulsion solvent extraction technique (16, 291, 304). For each batch of polyplex-loaded MPs, the aqueous polyplex solution was added to a mixture of PLGA and PEG dissolved in dichloromethane; this mixture was then vortexed to generate the first emulsion. For siRNA- or r-PEI-only loaded MPs, a portion of the aqueous siRNA or r-PEI stock solution was added to the PLGA/PEG mixture prior to the first emulsion. MPs loaded with r-PEI were prepared in aluminum-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 synthesis, 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, Fullerton, CA).
9.2.3. Quantification of entrapment efficiency

The entrapment efficiency of each MP batch was determined using a previously described protocol for siRNA-loaded polymeric MPs (303). Briefly, 15.0 mg of MPs were placed in 0.5 ml dichloromethane and incubated at room temperature until completely dissolved (1-2h). To extract the siRNA-PEI, siRNA, or r-PEI, 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, and then centrifuged at 11,000 × g for 5 min at 4°C. The aqueous phase was removed, the extraction repeated with 0.5 ml more of TE buffer, and the two aqueous phases were combined. The aqueous solution resulting from each MP formulation was assayed for either siRNA or r-PEI content according to the protocols described below. To determine the siRNA content of the aqueous extraction solutions from siRNA-PEI-loaded PLGA MPs, the polyplex dissociation protocol described below was used.

9.2.4. Polyplex release

For each PLGA MP batch, 20.0 mg samples (n = 4) were weighed out and suspended in 0.5 ml nuclease-free PBS (pH 7.4). All samples were incubated at 37°C on a shaker table at 70 rpm. At each timepoint (2h, 4h, 8h, 12h, and days 1, 2, 3, 4, 8, 12, 16, 19, 22, 25, 28), samples were centrifuged at 11,000 × g for 5 min at 4°C, the supernatant was removed and replaced with 0.5 ml fresh nuclease-free PBS, and the release vials were returned to 37°C. Collected supernatants were immediately stored at -20°C in a non-defrosting freezer to prevent degradation. Samples were briefly thawed and agitated
to remove aliquots for either siRNA or r-PEI assays as well as for dynamic light
scattering.

9.2.5. Quantification of r-PEI content

The amount of r-PEI in the release samples and in the aqueous solutions from
PLGA MP extraction was quantified according to a previously described protocol from
our laboratory (305), with a few modifications. The assay was prepared in opaque 96-
well plates by adding 100 μl of the standard or sample to each well and measuring the
fluorescence using a plate reader, with an excitation wavelength of 530 nm and an
emission wavelength of 620 nm. All samples and standards were run in triplicate.
Preliminary testing indicated that there was a significant difference in the fluorescence
emission of pure r-PEI and siRNA-r-PEI polyplexes. To account for these differences, r-
PEI samples were assayed using a standard curve (0-6 μg/ml r-PEI) comprised of the
material originally loaded into the MPs, i.e., either pure r-PEI or freshly prepared
polyplexes with either an 8:1 or 16:1 N:P ratio.

9.2.6. Polyplex dissociation and quantification of siRNA content

The siRNA content of the polyplexes was determined by adapting a protocol
previously described for plasmid DNA-PEI polyplex dissociation (306). Briefly, 50 μl of
each release sample or standard were added to the wells of an opaque 96-well plate. 100
μl of 0.5 v/v% PicoGreen dye (Molecular Probes, Eugene, OR) in alkaline TE buffer (pH
12, increased from pH 7.4 using 1N NaOH) was added to each well, and the plate was
immediately covered with aluminum foil and incubated for 5 min on a shaker table at 80
rpm. The fluorescence was then quantified immediately using a fluorescence plate reader,
with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. All samples and standards were run in triplicate. The standard curve consisted of freshly prepared siRNA-PEI polyplexes with either an 8:1 or 16:1 N:P ratio, diluted according to the amount of siRNA (range: 0-0.3 μg siRNA/ml PBS). Samples were diluted as necessary with nuclease-free PBS to fall within this range. Samples released from siRNA-only loaded MPs were not subjected to alkaline conditions; siRNA content was quantified at pH 7.4 using the PicoGreen assay according to the manufacturer’s instructions with a freshly prepared siRNA standard curve (0-4 μg siRNA/ml TE buffer (pH 7.4)).

9.2.7. Dynamic light scattering

Dynamic light scattering (DLS) analysis of release samples and freshly prepared polyplexes was performed using a 90PLUS particle size analyzer (Brookhaven Instruments, Holtsville, NY) operating at 659 nm wavelength and 37°C. For each formulation at each timepoint, all release samples (n = 3-4) were characterized. Samples were maintained at 37°C using an incubator and transferred one-by-one to the DLS for analysis. The intensity-weighted particle size distribution was derived from the accumulated autocorrelation function using the non-negatively constrained least squares (NNLS) algorithm. All calculations were performed using the BIC Dynamic Light Scattering software (Brookhaven Instruments) supplied with the DLS apparatus.

9.2.8. Polyplex stability

The stability of siRNA-PEI polyplexes was evaluated by preparing polyplexes with N:P ratios spanning the range of those observed for the release samples, incubating
the freshly prepared polyplexes at 37°C with gentle agitation, and measuring their
diameter via DLS after 4h and then again after 24h. The N:P ratios (listed in Figure 9.3)
and incubation times (4h and 24h) of the freshly prepared polyplexes were selected to
enable direct comparison with polyplexes released from PLGA MPs (Table 9.3).

9.2.9. Statistics

MP diameters are presented as mean ± standard deviation (n = 1500 particles per
MP formulation). Entrapment efficiencies are presented as mean ± standard deviation (n
= 3 samples). For each MP formulation, the release values are presented as a percentage
of the total measured amount of siRNA or r-PEI entrapped, determined by extracting the
siRNA, r-PEI, or polyplexes from a 15.0 mg sample of the PLGA MPs and quantifying
the amount of siRNA or r-PEI present. Data points for cumulative release of siRNA and
r-PEI, as well as hydrodynamic diameters of siRNA, PEI, and siRNA-PEI polyplexes,
represent mean ± standard deviation for (n = 4) samples at each timepoint. Cumulative
release and DLS data were analyzed using a repeated measures analysis of variance (p <
0.05), followed by Bonferroni post hoc analysis (p < 0.05). The effect of the two
independent factors (siRNA loading and N:P ratio) on the release profile was evaluated
using two-way analysis of variance (p < 0.05). For the freshly prepared polyplexes, the
effect of N:P ratio and incubation time (4h and 24h) on the diameter was evaluated via
two-way analysis of variance (p < 0.05), followed by Bonferroni post hoc analysis (p <
0.05) for multiple comparisons. The diameter of the freshly prepared polyplexes was
compared to that of the polyplexes from each release sample with equivalent N:P ratio
via analysis of variance (p < 0.05), followed by Bonferroni post hoc analysis (p < 0.05)
for multiple comparisons.
<table>
<thead>
<tr>
<th>Day</th>
<th>Low siRNA</th>
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<th>High siRNA</th>
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<tr>
<td></td>
<td>16:1 N:P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8:1 N:P</td>
<td>16:1 N:P</td>
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<td>0.1</td>
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<td>4 ± 1&lt;sup&gt;c&lt;/sup&gt;</td>
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<sup>a</sup>N:P ratios expressed with P set to 1. For instance, an N:P of 11:1 is expressed as “11.”
Calculated from the actual amount of siRNA and PEI detected from \((n = 4)\) PLGA MP release samples for this group at this timepoint. 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 \((C_2H_5N)\) 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 error for \((n = 4)\) siRNA and PEI measurements.

Value differs significantly from ideal N:P ratio of 8:1 \((p < 0.05)\).

Value differs significantly from ideal N:P ratio of 16:1 \((p < 0.05)\).

This N:P ratio was very large because the average amount of siRNA released was 1-2 orders of magnitude below all other release values, while the amount of PEI released was equivalent (in order of magnitude) to values at other timepoints for this group.

This N:P ratio could not be calculated because the average amount of siRNA released was 0.00 ± 0.07% of the total amount encapsulated, resulting in an N:P ratio with a zero-value denominator.

9.3. Results

9.3.1. Microparticle diameter

The MPs had a diameter of 26-34 \(\mu m\), and none of the formulations differed significantly from each other in size \((p > 0.05)\) (Table 9.1 and Table 9.2).

9.3.2. Entrapment efficiency of siRNA and PEI in the microparticles

The entrapment efficiency of siRNA (Table 9.1) differed significantly \((p < 0.05)\) amongst some of the PLGA MP formulations. Specifically, MPs with low siRNA theoretical loading \((-0.07 \mu g\) siRNA / mg PLGA MPs) had significantly higher siRNA entrapment efficiencies \((77 \pm 8\%\) for 16:1 N:P; 61 ± 13\% for 8:1 N:P; and 55 ± 5\% for
siRNA-only) than the MPs with high siRNA theoretical loading (~0.35 μg siRNA / mg PLGA MPs) (20 ± 4% for 16:1 N:P; 27 ± 5% for 8:1 N:P; and 39 ± 4% for siRNA-only) (Table 9.1). One of the groups with high siRNA theoretical loading (high siRNA, 16:1 N:P) also had a significantly lower (p < 0.05) PEI entrapment efficiency (51 ± 6%) than the other polyplex-loaded formulations (~72-75%; Table 9.2).

9.3.3. Release of siRNA from PLGA MPs

Each MP formulation had a unique siRNA cumulative release profile (p < 0.05) over 28 days (Figure 9.1a). The release followed a triphasic pattern consisting of an initial burst release for 24h, followed by a lag period, after which release resumed around day 12. For (siRNA-PEI) polyplex-loaded MPs, the total amount released during the burst period ranged from 6 ± 1% to 32 ± 2% of the siRNA initially encapsulated as a component of the polyplexes. The total burst release (52 ± 1%) from the “high siRNA only” loaded MPs was significantly higher (p < 0.05) than the amount released from all other MPs. Similarly, the 24h burst release from the “low siRNA only” MPs (29 ± 1%) significantly exceeded (p < 0.05) that of corresponding “low siRNA” formulations loaded with siRNA-PEI polyplexes (Figure 9.1a).

Main effects analysis (Figure 9.2) of the four polyplex-loaded formulations indicated that both siRNA loading and N:P ratio affected siRNA release. Loading had a significant effect on siRNA release at all times (p < 0.05), with a higher loading resulting in higher cumulative release. The two factors (siRNA loading and N:P ratio) significantly interacted (p < 0.05) during days 22-28, increasing release. The N:P ratio significantly (p < 0.05) affected release during the burst period and also on days 25-28. The N:P ratio had
opposing effects during these two time periods; a higher N:P ratio decreased release during the burst period, and increased release during days 25-28.

9.3.4. Release of PEI from PLGA MPs

For each polyplex-loaded formulation, the PEI and siRNA profiles differed. The PEI profiles had a 24h burst release period that was followed by gradual, approximately linear PEI release until day 28 (Figure 9.1b). The four polyplex-loaded MP formulations and the two PEI-only groups all had significantly different cumulative release profiles ($p < 0.05$) (Figure 9.1b). Burst release values ranged from $8 \pm 1\%$ to $72 \pm 10\%$ of the PEI initially encapsulated. For “high PEI only”, “high siRNA, 16:1 N:P,” and “high siRNA, 8:1 N:P,” the three formulations with the highest PEI loading (see values in Table 9.1), the vast majority of PEI release occurred during the burst period (at least $80 \pm 10\%$ of the amount released by day 28). For the other three formulations, burst release was a much smaller fraction of the overall PEI release; for the two polyplex-loaded formulations (“low siRNA, 16:1 N:P,” and “low siRNA, 8:1 N:P”), it was similar to the ratio of siRNA burst release compared to day 28 siRNA release: ~20\% of the day 28 value for “low siRNA, 16:1 N:P” and ~50\% of the day 28 value for “low siRNA, 8:1 N:P.”

Main effects analysis (Figure 9.2b) of the four polyplex-loaded formulations indicated that both siRNA loading and the N:P ratio significantly affected PEI release ($p < 0.05$) at all timepoints. The interaction of these two factors was also significant at all times ($p < 0.05$). Increasing the N:P ratio from 8:1 to 16:1 significantly increased PEI release, as did increasing the siRNA loading from ~0.07 to ~0.35 $\mu g$ siRNA / mg PLGA MPs ($p < 0.05$).
Figure 9.1: Release of siRNA and PEI from PLGA MPs over time

All PLGA MP formulations had unique (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 12. The PEI profiles (b) also had a 24h burst release period, followed by gradual, approximately linear PEI release until day 28. The four polyplex-loaded MP formulations and the two PEI-only groups all had significantly different cumulative release profiles ($p < 0.05$) (Figure 9.1b). Two sets of MPs were prepared for each polyplex-loaded formulation because quantification of siRNA and PEI content and release required the use of fluorometric assays that had overlapping emission and excitation wavelengths. MPs for siRNA measurements were loaded with siRNA-PEI polyplexes, while MPs for PEI measurements were loaded with polyplexes containing rhodamine-labeled PEI. Values for each group are expressed as a percentage of the amount of siRNA or PEI initially encapsulated (entrapment efficiency × theoretical loading from Table 9.1 and Table 9.2). Data points represent the mean ± standard deviation for $n = 4$ samples, each consisting of 20 mg of MPs suspended in 0.5 ml PBS and incubated at 37°C with gentle agitation. Error bars are included for all groups, though they are too small to resolve in some cases.
Main effects analysis of the polyplex-loaded PLGA MPs indicated that siRNA loading and the N:P ratio affected release of both (a) siRNA and (b) PEI. The vertical axis represents the change in cumulative (a) siRNA or (b) PEI release that results when either of the two factors (N:P ratio or siRNA loading) is changed from the low to the high.
value. Increasing siRNA loading from \( \sim 0.07 \) to \( \sim 0.35 \, \mu g \, \text{siRNA} / \, \text{mg PLGA MPs} \) resulted in significantly higher (a) siRNA and (b) PEI cumulative release at all timepoints \((p < 0.05)\). Increasing the N:P ratio from 8:1 to 16:1 resulted in (a) significantly lower siRNA cumulative release \((p < 0.05)\) during the burst period (2h to day 1), and increased siRNA release during days 25-28 \((p < 0.05)\). Increasing the N:P ratio significantly increased PEI cumulative release at all timepoints \((p < 0.05)\). A positive value indicates that increasing the factor from the low to the high value resulted in an increase in cumulative release. The error bars represent the standard error of the mean for \( n = 4 \) samples.

### 9.3.5. N:P ratio of the polyplexes released from PLGA MPs

A repeated measures analysis of the calculated N:P ratios of the polyplexes released at each timepoint (Table 9.3; "N" values reported after setting "P" to 1 for each calculation) did not indicate any significant differences amongst the groups over time \((p > 0.05)\). However, comparison of the calculated N:P ratios to the ideal values (either 16:1 or 8:1) indicated significant differences during the burst period (days 0-1) and also in the days following the siRNA lag period (i.e., days 16-28) (Table 9.3). The “low siRNA, 8:1 N:P” and “high siRNA 8:1 N:P” groups had N:P ratios significantly less than 8:1 \((p < 0.05)\) at 2h (0.1 days) and significantly greater than 8:1 \((p < 0.05)\) at 8h (0.3 days). The “low siRNA, 16:1 N:P” group had calculated N:P ratios significantly less than 16:1 \((p < 0.05)\) at 8h (0.3 days), 12h (0.5 days), and on day 1. The “high siRNA, 16:1 N:P” group had calculated N:P ratios significantly greater than 16:1 \((p < 0.05)\) at 4h (0.2 days) and 8h (0.3 days). On days 19-28, all groups had calculated N:P ratios that were much less \((p < 0.05)\) than their respective ideal values. Groups with the same ideal N:P ratio had similar calculated N:P values during this time (Table 9.3).
9.3.6. Size and stability of freshly prepared siRNA-PEI polyplexes

The particle diameters measured via DLS were separated into three size ranges: <50 nm, 50-250 nm, or >300 nm, based on the peaks observed for pure PEI and pure siRNA. DLS analysis of the pure polyplex components in PBS indicated that pure PEI contained a single population of particles whose diameter (6.4 ± 0.3 nm) was significantly smaller ($p < 0.05$) than that of either of the two populations (52 ± 10 nm and 360 ± 50 nm) observed for pure siRNA (Figure 9.3a – top row). With the exception of the freshly prepared 581:1 N:P formulation, for which DLS only detected <50 nm particles that were equivalent in diameter to pure PEI ($p > 0.05$) (Figure 9.3b), all of the freshly prepared polyplex samples had a population of particles in the 50-250 nm range (Figure 9.3b – middle panel). These particles were typically ~150 nm in diameter and, in most cases, their size significantly differed from pure PEI and from both of the pure siRNA peaks ($p < 0.05$), suggesting this population consisted of siRNA-PEI polyplexes. Most polyplexes were stable over time, except for those with 17:1 and 73:1 N:P ratios, which approximately tripled in diameter from 4h to 24h (Figure 9.3b – middle panel), resulting in the release of free PEI (Figure 9.3b – upper panel). Large (>300 nm) particles were only detectable in the fresh polyplex samples with the smallest N:P ratios (1:1 and 4:1), while free PEI appeared as the N:P ratio increased to 15:1 and above (Figure 9.3b - upper panel). The intensity of the peaks corresponding to free PEI increased as the surplus of PEI (i.e., the N:P ratio) increased (Figure 9.3a – third row vs. bottom row).
Figure 3a – figure continued on next page
Figure 9.3: Size and stability of freshly prepared siRNA-PEI polynplexes
Freshly prepared polynplexes with varying N:P ratios (from 1:1 to 581:1) had similar hydrodynamic diameters via DLS after 4h and 24h incubation at 37°C. Many samples contained more than one population of particles (multiple peaks) (a). The diameters were separated into three ranges: <50 nm, 50-250 nm, or >300 nm (b), based on the peaks observed for pure PEI (“PEI only”; 6.4 ± 0.3 nm) and pure siRNA (“siRNA only”; 52 ±
10 nm and 360 ± 50 nm), all of which differed significantly ($p < 0.05$). With the exception of the 581:1 N:P formulation, for which DLS only detected free PEI (upper panel of (b)), all of the formulations had polyplexes ~150 nm in diameter (middle panel of (b)); for most formulations, polyplex size differed significantly from both PEI and siRNA ($p < 0.05$) (indicated by *). Most polyplexes were stable over time; # indicates peaks that differed significantly ($p < 0.05$) at 24h compared to 4h. All <50 nm particles (upper panel of (b)) were equivalent in size to pure PEI ($p > 0.05$) and differed significantly from pure siRNA ($p < 0.05$). Representative 37°C DLS curves are shown in (a), while the bar charts (b) summarize the average hydrodynamic diameter ± standard deviation for all ($n = 3$) samples.

### 9.3.7. Size of siRNA-PEI polyplexes released from PLGA MPs

All release samples from the siRNA-PEI-loaded PLGA MPs contained a population of particles in the 50-250 nm range (Figure 9.4a), consistent in size with the freshly-prepared siRNA-PEI polyplexes (Figure 9.3b – middle panel). The polyplexes released from PLGA MPs showed little variation in diameter despite large variations in N:P ratio (Table 9.3). A repeated measures analysis of the average polyplex diameter from each group indicated that the “low siRNA, 16:1 N:P” formulation resulted in polyplexes of significantly different diameter over time ($p < 0.05$) (overall average diameter of 120 ± 50 nm compared to 140 ± 50 nm for the 8:1 N:P formulations and 140 ± 60 nm for “high siRNA, 16:1 N:P”) (Figure 9.4b). In addition to the polyplexes, many formulations contained a variety of larger (500-1000 nm) particles at various timepoints; in most cases, the diameter of these particles did not differ ($p > 0.05$) from that of the PLGA MP derivatives (including small MPs and PLGA degradation products) observed in the release medium collected from ($n = 3$) non-drug-loaded MPs (gray shading in Figure 9.4a). Most release samples from siRNA-PEI-loaded PLGA MPs did not contain particles as small as free PEI or free siRNA (i.e., of diameter <100 nm); if they were
detected (e.g., both “low siRNA” formulations at 4h in Figure 9.4a,b), such small particles were significantly larger ($p < 0.05$) than free PEI but did not differ ($p > 0.05$) in size compared to the smaller ($52 \pm 10$ nm) free siRNA particles.

The released siRNA-PEI polyplexes (Figure 9.4) were similar in size to the freshly prepared samples with equivalent N:P ratios (Figure 9.3), with a few exceptions. Differences were mainly observed in samples from the burst release period. At the 12h timepoint (Figure 9.4b), the “low siRNA, 8:1 N:P” and “high siRNA, 16:1” formulations contained polyplex-sized particles (140 ± 20 nm and 140 ± 30 nm, respectively), while freshly prepared samples with equivalent N:P ratios (1:1 and 73:1, respectively) and 4h incubation time contained smaller particles (82 ± 15 nm and 43 ± 10 nm, respectively) equivalent ($p > 0.05$) to the diameter of free siRNA. The “low siRNA, 8:1 N:P” day 4 sample, which had the largest calculated N:P ratio (581:1; Table 9.3) contained 140 ± 50 nm diameter particles, much larger than the freshly prepared 581:1 N:P samples after either 4h or 24h (Figure 9.3), where only free-PEI-sized particles (5.6 ± 1 nm) were detectable via DLS. The converse was also observed; the “high siRNA, 8:1 N:P” release samples from days 2 and 28 (both with 4:1 calculated N:P ratios; Table 9.3), as well as the “low siRNA, 8:1 N:P” 4h samples (with 7:1 calculated N:P), contained particles with diameter equivalent to free siRNA (53-86 ± 30 nm) that were smaller ($p < 0.05$) than the ~150 nm polyplexes seen in fresh samples with equivalent N:P ratios. The “low siRNA, 16:1 N:P ratio” 12h release samples presented a unique case: the 95 ± 13 nm particles detected (Figure 9.4b) were significantly smaller ($p < 0.05$) than fresh polyplexes of equivalent 4:1 N:P ratio, but larger than free siRNA ($p < 0.05$).
Figure 10.4a – figure continued on next page
Figure 9.4: Size of siRNA-PEI polyplexes released from PLGA MPs

Samples released from siRNA-PEI polyplex-loaded PLGA MPs contained more than one population of particles (multiple peaks) as detected via DLS (a). All samples contained a population of particles that were <300 nm in size. In most cases, the smallest particles were ~150 nm in diameter and were significantly larger ($p < 0.05$) than pure PEI ("PEI only"; 6.4 ± 0.3 nm) and intermediate in size between the two populations of pure siRNA particles ("siRNA only"; 52 ± 10 nm and 360 ± 50 nm). The larger particles typically corresponded to those in the release media collected from non-drug-loaded PLGA MPs (gray shading in (a)). Representative DLS curves at 37°C are shown in (a) for each formulation at each timepoint; 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. The charts in (b) summarize the average hydrodynamic diameter ± standard deviation of the smallest particles for all ($n = 4$) siRNA-PEI loaded PLGA MP samples. Each inset chart shows a detailed view of the DLS data from the first few timepoints, which were separated by only several hours. The formulation marked with # had particles with significantly different hydrodynamic diameter ($p < 0.05$) compared to the other formulations, determined via a repeated measures analysis of DLS data from all timepoints.
9.4. Discussion

This study describes the development of a sustained release system for rat anti-TNF-α siRNA for potential use as an intra-articular controlled release system for treatment of TMJ inflammation. The delivery system described herein has the potential to deliver anti-TNF-α siRNA over a period of several weeks following a single intra-articular injection, minimizing the risk of iatrogenic TMJ injury.

The incorporation of PEI, a polymeric transfection agent, into the design of the siRNA delivery system offers several advantages over delivery of siRNA alone (295). In addition to protecting siRNA from degradation by ubiquitous extracellular nucleases and promoting cellular uptake of released siRNA (296), we anticipate that PEI will eliminate the need for joint electroporation (276, 277) to achieve transfection. In a recent short-term study, a single intra-articular injection of siRNA-PEI effectively treated painful TMJ inflammation for 48h in our intended rat model. The anti-inflammatory siRNA was internalized within cells of the rat TMJ tissue without the need for electroporation (307). A recent study of siRNA-loaded PLGA nanoparticles and MPs indicated that addition of a transfection agent was necessary to achieve in vitro gene silencing (299), while another reported that siRNA-PEI-loaded PLGA MPs achieved superior in vivo gene silencing compared to siRNA-only-loaded MPs (298). In the present work, PEI modulated siRNA release from PLGA MPs (Figure 9.1a), decreasing burst release and acting as a porogen to facilitate later siRNA release. This was particularly evident for siRNA release from the “high siRNA, 16:1 N:P” formulation during days 16-28 (Figure 9.1a), which had the highest PEI loading (Table 9.2).
Two sets of MPs were prepared for each formulation because quantification of siRNA and PEI content and release required the use of fluorometric assays that had overlapping emission and excitation wavelengths. 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). The yield of r-PEI-loaded PLGA MPs was lower than that of MPs with unlabeled PEI (e.g., 69 ± 1% versus 81 ± 3% for “low siRNA, 16:1 N:P”) (Table 9.1 and Table 9.2). This likely resulted from reduced visibility during MP fabrication due to the aluminum foil wrapping and dim lighting conditions necessary to prevent quenching of the rhodamine dye. As described previously (16, 291), in the final steps of fabrication, MPs are rinsed with water several times, and collected via centrifugation. They are then flash-frozen in liquid nitrogen and subsequently lyophilized. For r-PEI-loaded MPs, the water is aspirated following each of the rinse steps without being able to see the MPs at the bottom of the centrifuge tube (due to the foil wrapping and dim lighting) (16, 291). Thus, it is likely that more r-PEI-loaded MPs were accidentally aspirated during these wash steps than during the analogous steps for non-fluorescent MPs, resulting in final PLGA MP yields that were lower for MPs with r-PEI (Table 9.2) than those with unlabeled PEI (Table 9.1). In contrast, PLGA MP diameter, a visibility-independent parameter, did not significantly vary based on the incorporation of PEI or r-PEI (e.g., 31 ± 2 µm versus 32 ± 4 µm for “low siRNA, 16:1 N:P” with PEI and r-PEI, respectively) (Table 9.1 and Table 9.2). We assumed that the presence of rhodamine (in r-PEI) did not significantly affect polyplex entrapment or release, due to the very low molecular weight of the rhodamine dye (413 Da) compared
to that of the PEI (~25 kDa), as well as because of the small amount of dye used (~1 mol dye per 100 mol of primary amines on the PEI).

For both siRNA and PEI, the decrease in entrapment efficiency with increasing loading (Table 9.1 and Table 9.2) likely stemmed from the fact that higher loading was achieved by using a larger volume of aqueous stock solution; since the MPs were synthesized using the water-oil-water double emulsion method, this larger volume of the internal aqueous phase likely promoted leaching of the siRNA into the external aqueous phase during MP formation. The “high siRNA, 16:1 N:P” formulation had the highest theoretical loading of PEI (0.81 ± 0.05 µg / mg PLGA MPs), twice as great as the next highest theoretical loading (0.42 ± 0.00 µg PEI / mg PLGA MPs for the high siRNA, 8:1 N:P group). The significantly lower (p < 0.05) PEI entrapment efficiency (Table 9.2) of “high siRNA, 16:1 N:P” compared to the other polyplex-loaded PLGA MP groups suggests that the combined volume of siRNA and PEI stock solutions necessary to create this PEI loading was large enough that PEI also leached out during MP fabrication.

The various drug loading parameters did not significantly affect the diameter of the PLGA MP formulations (p > 0.05; Table 9.1 and Table 9.2), which approximated that of blank PLGA MPs (22 ± 7 µm) used in a previous study demonstrating their in vivo biocompatibility when injected intra-articularly into the rat TMJ (16). In that study, the maximal amount of blank PLGA MPs that could be suspended and reproducibly injected was ~2.5 mg per TMJ (16). To achieve an equivalent amount of siRNA to that injected as a free drug solution in the in vivo rodent knee electroporation studies (276, 277) and in the recent TMJ study (307), PLGA MP loading would need to be one order of magnitude higher than that of the “high siRNA” PLGA MP formulations (Table 9.1). However, in
*vivo* efficacy of siRNA-PEI-loaded PLGA MPs has been reported for MPs with much lower loading (298), suggesting that improved retention at the target site and controlled siRNA release over time reduce the required dose.

The low and high siRNA loading values (~0.07 and ~0.35 μg siRNA/mg PLGA MPs; Table 9.1) were selected from the range of values (~0.0003 to 0.4 μg siRNA/mg PLGA) reported in previous studies of siRNA- and (siRNA-PEI)-loaded PLGA MPs and nanoparticles. Although previous studies of PLGA MPs have utilized much lower siRNA loading values (~0.0003-0.003 μg siRNA/mg PLGA MPs) (297, 298), the total amount of MPs used to achieve *in vivo* efficacy was much higher (~10 mg) (298) than the amount (~2.5 mg; see previous discussion) that can be delivered to our anticipated *in vivo* TMJ model. As a result, we selected higher siRNA loading values to compensate for the lower PLGA delivery amount. This strategy is consistent with the results of a previous study of (siRNA-PEI)-loaded poly(DL-lactic acid) (PLA) MPs (303). Injection of 3 mg of PLA MPs (loaded with 0.2 μg siRNA/mg MPs) effectively treated an *in vivo* model of peritoneal inflammation (303). The loading value used for the PLA MPs falls within the range (0.2-0.4 μg siRNA/mg PLGA) reported for siRNA-loaded PLGA nanoparticles (299, 302). Consequently, we chose the upper limit of this range as the higher siRNA loading value (~0.35 μg siRNA/mg PLGA MPs; Table 9.1), and then arbitrarily set the lower siRNA loading value to ~0.07 μg siRNA/mg PLGA MPs.

Although PLGA MP degradation results in an acidic microenvironment (308), conditions within the MPs in this study did not compromise siRNA structure. Approximately 50-80% of the siRNA initially encapsulated in the siRNA-only loaded MPs (Figure 9.1a) was released by day 28. Since the detection limit of the PicoGreen dye
(20 base pairs (309)) is approximately equivalent to the size of the siRNA (21 base pairs), all siRNA detected in our studies was intact, i.e., neither degraded nor denatured. These results are supported by published gel electrophoresis of siRNA encapsulated in PLGA nanoparticles and MPs (298, 301).

To our knowledge, this is the first report of the successful dissociation of siRNA-(branched PEI) polyplexes in order to quantify siRNA content. To date, accurate measurement of siRNA content in siRNA-PEI polyplexes has been a major challenge in the field because siRNA is much smaller than plasmid DNA, and thus PEI entirely blocks binding of fluorescent siRNA-binding dyes (PicoGreen, ethidium bromide, etc.) (296, 310). Several studies have reported the use of fluorescent siRNA-binding dyes to quantify siRNA content in siRNA-PEI polyplexes (303, 311), without specifying whether siRNA was dissociated from PEI. Another study reported that the siRNA content of siRNA-PEI-loaded PLGA MPs was quantified by HPLC and visualized via gel electrophoresis of the release medium (298), again without specifying whether siRNA was dissociated from PEI. It has been shown that gel electrophoresis of siRNA-(linear PEI) requires polyplex dissociation via the addition of heparan sulfate, otherwise siRNA-PEI aggregates remain trapped in the wells and do not migrate within the gel (312).

The dissociation technique described in the present work enabled accurate measurement of unlabeled siRNA released as a component of siRNA-(branched PEI) polyplexes, and was developed by adapting a technique from the plasmid DNA literature. Addition of heparin and increase in pH are two established methods for disrupting the electrostatic bonds in plasmid DNA-PEI polyplexes (306). Since RNA is more susceptible to base-catalyzed degradation than DNA (296, 313), we initially attempted
siRNA-PEI polyplex dissociation via the addition of anionic heparin. Although incubation of freshly prepared siRNA-PEI polyplexes (8:1 and 16:1 N:P) with 6.3, 12.5, and 25 mg/ml heparin (5000 USP units/ml; American Pharmaceutical Partners, Schaumburg, IL) successfully dissociated freshly prepared 8:1 N:P polyplexes, it failed to dissociate siRNA-PEI with a 16:1 N:P ratio (data not shown; protocol for 12.5 mg/ml heparin and DNA-PEI polyplexes is detailed in (306)). The successful dissociation of the siRNA-(branched PEI) polyplexes with an 8:1 N:P ratio was consistent with a previous report of the dissociation of 5:1 N:P siRNA-(linear PEI) polyplexes with a related molecule, heparan sulfate (312). However, dissociation of siRNA-PEI polyplexes with higher N:P ratios was not described in that study (312).

The 8:1 and 16:1 N:P ratios used for the various PLGA MP formulations (Table 9.1) were selected from the range of values reported for effective in vitro and in vivo gene silencing. While some publications have reported effective gene silencing by siRNA-PEI polyplexes with 6:1 or 8:1 N:P ratio, others have reported that higher ratios were required (298, 300, 303, 307, 310). A recent study of PLA MPs encapsulating polyplexes consisting of murine anti-TNF-α siRNA and 25 kDa branched PEI (303), demonstrated that polyplexes with very low N:P ratio (i.e., 4:1) were not entrapped in PLA MPs fabricated via a technique similar to that used to generate our PLGA MPs. N:P ratios of 8:1 and 16:1 resulted in siRNA entrapment similar to that reported in Table 9.1, and 8:1 N:P polyplexes released from the PLA MPs effectively treated an in vivo model of peritoneal inflammation (303). Since the efficacy of gene silencing varies according to siRNA sequence and target cell type (314), the optimal N:P ratio for our intended TMJ model will ultimately be determined by in vivo evaluation of the formulations described
herein. For this reason, we did not wish to abandon the 16:1 N:P formulations at this preliminary stage, despite the difficulty in dissociating the siRNA and PEI.

Since we were unable to dissociate the 16:1 N:P polyplexes using heparin, we abandoned this approach and attempted dissociation via alkaline pH. In contrast to heparin, elevated pH successfully dissociated polyplexes with both 8:1 and 16:1 N:P ratios, and the siRNA content was successfully determined prior to siRNA degradation or denaturation by the alkaline environment. Our findings of siRNA stability for the ~10 min period of time necessary to perform the alkaline pH PicoGreen assay are supported by a previous study of base-catalyzed RNA degradation using RNA of similar length; <1% of the naked RNA degraded under similar conditions (pH, ionic strength, exposure time, and temperature) (313). Dissociation of the polyplexes enabled measurement of the cumulative release of each component (Figure 9.1), which in turn enabled calculation of the approximate N:P ratio at each timepoint (Table 9.3; “N” values reported after setting “P” to 1 for each calculation). These calculations were performed assuming that the presence of rhodamine (in r-PEI) did not significantly affect polyplex release, so that the measured r-PEI release from siRNA-r-PEI-loaded PLGA MPs could be used to approximate the PEI release from siRNA-PEI-loaded PLGA MPs.

The differing shapes of the PEI and siRNA cumulative release curves (Figure 9.1) suggest that, despite being loaded into MPs as polyplexes, siRNA and PEI were separately released and then assembled into polyplexes upon exiting the MPs. Main effects analysis of the siRNA and PEI release data (Figure 9.2) elucidated the individual contributions of the two factors, siRNA loading and N:P ratio, on release at each timepoint. To our knowledge, such an analysis has not yet been presented in the literature.
describing delivery of siRNA-PEI polyplexes. During the initial 24h burst period, both factors significantly affected \( p < 0.05 \) release of both siRNA and PEI. Higher siRNA loading and higher N:P ratio, both of which indirectly increased the PEI loading (Table 9.2), resulted in increased PEI burst release (Figure 9.2b). Both factors continued to significantly affect \( p < 0.05 \) PEI release throughout the study. The influence of nucleotide loading on PEI release is consistent with a previous study of coaxial microfibers consisting of a PEG core loaded with plasmid DNA and a poly(ε-caprolactone) sheath containing hyaluronic-acid-functionalized r-PEI (305). As the PEI and DNA were released from the microfibers, they assembled into polyplexes. Of the four factors examined in the study (sheath and core polymer concentrations, PEG molecular weight, and DNA concentration), plasmid DNA concentration was the only factor that significantly affected PEI release. Unlike the current study, the N:P ratio was kept constant and thus not examined via main effects analysis (305). In contrast to PEI, siRNA release was mainly influenced by siRNA loading, with the N:P ratio contributing to release only during the burst period and days 25-28. Increasing the N:P ratio had opposing effects during these two periods. while a higher N:P ratio promoted siRNA retention within the PLGA MPs during the burst period, it increased siRNA release at the end of the study (Figure 9.2a). The information gained from the main effects analysis will be useful in the design of future siRNA-PEI-loaded PLGA MP delivery systems.

During days 1-12, the calculated N:P ratios (Table 9.3) reached very high values (e.g., 243 ± 150 and 581 ± 500 on day 4 for “low siRNA, 16:1” and “low siRNA, 8:1,” respectively) as a result of the lag phase in siRNA release (Figure 9.1a) occurring at the same time as continued, nearly linear PEI release (Figure 9.1b). Although the calculated
N:P ratio varied greatly, from 1:1 to 581:1 (Table 9.3), it is likely that the amount of PEI associated with siRNA spanned a much smaller range. DLS analysis of freshly prepared polyplexes indicated that N:P ratios of 15:1 or higher resulted in detectable free PEI particles (Figure 9.3). In contrast, release samples with high calculated N:P ratios did not contain detectable free PEI, with the exception of the sample with the highest calculated N:P ratio (581 ± 500; Table 9.3); the “low siRNA, 8:1 N:P” sample from day 4 had a small free PEI peak (Figure 9.4a). The absence of free PEI in the release samples suggests that the cationic PEI was complexed with PLGA MP derivatives, including small MPs and degradation products, which contain anionic carboxyl groups at physiologic pH (298). This sequestration of PEI is beneficial, since cytotoxicity is a major limitation of PEI as a siRNA transfection vehicle (295, 310, 315, 316). Complexation of excess PEI with anionic PLGA MP derivatives will reduce its interaction with negatively-charged cell membranes and thus limit its potential in vivo cytotoxicity (315). Notably, the only study that has examined the effect of siRNA-PEI in an inflamed TMJ animal model did not report any adverse effects of siRNA-PEI compared to PEI alone (307).

This study reports the hydrodynamic diameter of siRNA and siRNA-PEI polyplexes under simulated physiologic conditions (37°C, PBS buffer, pH 7.4). Pure siRNA samples contained two different populations of particles (52 ± 10 nm and 360 ± 50 nm; Figure 9.3a), likely single molecules and aggregates of several siRNA molecules, respectively. The size of the larger siRNA population was a multiple (7 ± 2 ×) of the smaller diameter, suggesting that this peak resulted from aggregated siRNA. In contrast to DNA-PEI polyplexes, in which condensation of the large plasmid DNA is observed,
resulting in polyplexes smaller than pure DNA, the siRNA-PEI polyplexes (~150 nm diameter) were larger than siRNA alone (Figure 9.3a and Figure 9.4a). This finding is consistent with biophysical theory, which states that the short length of siRNA (21 base pairs, which is shorter than the persistence length of RNA) inhibits chain flexibility and causes the molecule to behave as a "rigid rod." Thus, siRNA is not condensed by PEI, unlike plasmid DNA, which is typically several orders of magnitude longer than siRNA and is thus much more flexible (296). The presence of pure siRNA aggregates (>300 nm particles; Figure 9.3a,b) and the persistence of these larger particles in the presence of low amounts of PEI (as for the 1:1 and 4:1 N:P ratios) (Figure 9.3b) are consistent with a phenomenon that has been recently described in detail via DLS analysis of plasmid DNA-PEI polyplexes (317). Initially, transient aggregates of siRNA form. Addition of small amounts of PEI results in siRNA-PEI polyplexes that are prone to aggregation due to charge imbalance. With the addition of more PEI (i.e., raising the N:P ratio) the large particles disappear as charge repulsion results in the formation of dispersed siRNA-PEI polyplexes.

We found that varying the N:P ratio did not dramatically affect siRNA-PEI diameter, which remained at ~150 nm (Figure 9.3). Few studies have examined the impact of N:P ratio on the size of siRNA-PEI polyplexes (310, 316, 318). Two published studies that utilized DLS to measure siRNA-PEI hydrodynamic diameter have reported conflicting results. One group reported that varying the N:P ratio of siRNA-(linear 25 kDa PEI) from 5:1 to 90:1 decreased polyplex diameter from >200 nm to ~50 nm. For N:P ratios ranging from 15:1 to 60:1, the polyplex diameter remained constant at ~150 nm (318). However, the opposite trend was reported for siRNA-(branched 25 kDa PEI)
polyplexes; those with 7:1 N:P ratio had a hydrodynamic diameter of 170 nm, while 15:1 N:P polyplexes were much larger (450 nm) (310). Several factors may explain the differences between these published reports and our data, including the structure of the PEI (linear vs. branched), the buffer used, and, most importantly, the temperature-dependence of DLS measurements. Hydrodynamic diameter is determined using equations that depend upon sample temperature and on temperature-dependent values like viscosity. In both of these published studies, DLS was performed at 25°C (310, 318), compared to 37°C in this work. A difference of 25°C vs. 37°C has been previously shown to significantly impact the hydrodynamic diameter of plasmid DNA-PEI polyplexes (319). We selected the higher temperature (37°C), as well as PBS as a buffer, so that DLS measurements would reflect particle size in vivo.

The consistent, small (~150 nm) diameter of the siRNA-PEI polyplexes released from PLGA MPs over the entire 28-day length of this study (Figure 9.4b) is particularly exciting because it maximizes the potential intracellular uptake of the polyplexes (296, 316). A recent study reported that siRNA-PEI polyplexes larger than ~150 nm were unable to induce gene silencing in vitro. Size was not the only factor governing transfection efficiency; the other important parameters were polyplex stability and the N:P ratio, because of its effects on surface charge (316). Based on these promising results, future studies will evaluate these siRNA-PEI-loaded MPs in a rat TMJ disorder model (19, 307) to determine their in vivo efficacy in alleviating TMJ inflammation.
9.5. Conclusion

We have demonstrated the synthesis of distinct PLGA MP formulations loaded with siRNA, PEI, or siRNA-PEI polyplexes. Systematic variation of siRNA loading and N:P ratio resulted in distinct patterns of siRNA and PEI sustained release over 28 days. A novel method for dissociating the siRNA-PEI, presented herein, enabled calculation of the N:P ratio of polyplexes released over time. Despite large variations in N:P ratio, the diameter of the release polyplexes showed little variation, remaining within established size limits for intracellular uptake. Taken together, these results underscore the exciting potential of these PLGA MP formulations as the basis for the first intra-articular controlled release system for treating TMJ disorders.
Chapter 10

Intra-Articular Controlled Release of Anti-Inflammatory siRNA Ameliorates Temporomandibular Joint Inflammation

10.1. 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 (12). In severe TMJ degeneration, oral medications are often insufficient for pain relief, and thus intra-articular injections of corticosteroids or hyaluronic acid are necessary to treat pain. However, these intra-articular formulations are complicated by rapid clearance of injected agents, which necessitates frequent injections that carry a high risk of iatrogenic injury (12, 13). These
limitations have motivated research into the development of novel therapeutics, including sustained release delivery systems, to alleviate severe TMJ pain.

Biodegradable poly(DL-lactic-co-glycolic acid) (PLGA) microparticles (MPs) have been used for a wide variety of drug delivery applications. Recent reports have demonstrated the in vitro controlled release of small interfering RNA (siRNA) from PLGA MPs (17, 297, 298), and one report has described the efficacy of PLGA-based MPs in delivering siRNA to treat tumors in vivo (298). Motivated by these results, we have been developing a PLGA MP-based system for intra-articular delivery of siRNA-PEI to the TMJ. We have previously reported that intra-articular PLGA MPs are biocompatible in vivo in the rat TMJ (16) and have also demonstrated the ability of PLGA MPs to provide sustained release of polyplexes consisting of siRNA together with the transfecting agent polyethylenimine (PEI) (17).

Recently, inflammatory cell signaling via the cell-surface Fc receptor for immunoglobulin type G (IgG) (FcγRIII, also known as CD16) was implicated as a significant factor in painful TMJ inflammation (307). Silencing of FcγRIII signaling via intra-articular injection of siRNA targeting FcγRIII, using PEI as a transfecting agent, showed promising results in rat model of TMJ inflammation for several days (307). 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 (274, 275).

In this study, we loaded PLGA MPs with anti-FcγRIII-siRNA-PEI polyplexes and evaluated the ability of this delivery system to treat painful TMJ inflammation in a rat model. To our knowledge, this is the first intra-articular controlled release system for the
The objective of this study was to evaluate the therapeutic efficacy of various doses of anti-FcγRIII-siRNA-PEI-loaded PLGA MPs as the first intra-articular sustained release system for TMJ inflammation.

10.2. Materials and Methods

10.2.1. Experimental Design

A full factorial design was used to evaluate the effect of anti-inflammatory siRNA-PEI polyplexes and PLGA MP dosage on TMJ inflammation. The two factors were MP dose (low or high) and siRNA-PEI loading into the MPs (polyplexes present or absent). The “Low anti-FcγR3 siRNA MPs” and “High anti-FcγR3 siRNA MPs” groups received low (1.5 mg per joint) and high doses (2.3 mg per joint) of siRNA-PEI-loaded PLGA MPs, respectively. The two polyplex-free groups, “Low blank MPs” and “High blank MPs,” received the corresponding amount of empty PLGA MPs.

Although several inflammatory mediators, including TNF-α and IgGs are elevated in the TMJs of patients and animal models with painful inflammation (18-20), injection of these agents does not reproduce the severe inflammation seen in TMJ disorders. For example, intra-articular injection of TNF-α yields only mild inflammation in animal models, requiring the injection of an irritant to establish an animal model of joint inflammation (21). In this study, we used Complete Freund’s Adjuvant (CFA), a severely irritating water-in-oil emulsion containing killed Mycobacterium tuberculosis and paraffin oil, to induce inflammation in the rat TMJ. This is an established, optimized method of creating a model of TMJ inflammation (22-27). A “CFA only” control group
was included in the design, which was not injected with MPs. All groups were injected with pro-inflammatory CFA to induce TMJ inflammation.

10.2.2. 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 one of the five treatment groups (n = 9 rats per group) and housed individually in cages equipped with previously described photobeam computer-activated pellet feeders (16, 307). 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, creating a record of meal patterns over time.

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 isoflurane gas (5% v/v in oxygen). The rats received bilateral 50 μl intra-articular TMJ injections consisting of PLGA MPs (50 or 75 mg/ml) in 10% v/v Tween 20 (J.T. Baker, Phillipsburg, NJ) in normal saline. An experienced individual (Dr. Phillip R. Kramer of Baylor College of Dentistry, Dallas, TX) performed the injections using an established approach that reliably delivers the MPs to the superior joint space of the TMJ (16). 72h after this initial injection, all rats were anesthetized and received bilateral 20 μl TMJ injections containing 15 μg CFA (Chondrex, Redmond, WA), an established method to induce painful TMJ inflammation (307). Meal parameters, including meal duration and food intake, were recorded for a total of 9 days (including the 2 day
baseline) using the computer-activated pellet feeders. Established, validated threshold values for minimum meal size and meal duration were input into the meal pattern software, as previously described (307).

Seven days after MP injection, each rat was removed from its cage, taken into a different room, and euthanized within 20 s by decapitation. To obtain samples for the ELISAs and Western blot, the TMJ and soft tissue were dissected according to an established protocol (307), and then flash-frozen in liquid nitrogen and stored at -80°C until protein isolation was performed. Samples for immunohistochemistry were obtained as previously described (16, 307). Briefly the TMJ region was removed en bloc, fixed in 4% w/v paraformaldehyde in PBS for 48h, demineralized in 0.5 M EDTA using a Biowave® microwave tissue processing system (Pelco, Redding, CA) for 2 weeks, and then submerged in 25% w/v sucrose for 24h. The processed tissue was embedded in freezing medium and 20 µm thick sagittal sections were cut using a cryotome (Damon/IEC, Needham Heights, MA) and placed on Superfrost Plus slides (StatLab, Lewisville, TX).

10.2.3. Immunohistochemistry

The slides were blocked for 1h at room temperature in phosphate-buffered saline (PBS) containing 5% v/v goat serum and 0.3% v/v Triton X-100, and then incubated overnight at 4°C in PBS containing 1% w/v bovine serum albumin, 0.3% Triton X-100, and 1:100 diluted anti-CD16 and anti-CD14 primary antibodies (sc-20627 and sc-5749, respectively; both from Santa Cruz Biotechnology, Santa Cruz, CA). Next, the slides were rinsed three times (5 min each) in PBS containing 0.3% v/v Triton X-100, and then incubated for 1h at room temperature protected from light in PBS containing 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, Molecular Probes, Carlsbad, CA). After three rinses in 0.3% v/v Triton X-100 in PBS (10 min each), the slides were mounted using Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA) to which Hoechst dye had been added as a counterstain, and imaged using a fluorescence microscope (Nikon Instruments, Melville, NY).

10.2.4. Protein Extraction and ELISAs

For protein extraction, 1 ml TPER lysis buffer (Thermo Scientific, Waltham, MA) containing 1% v/v HALT protease inhibitor (Thermo Scientific) was added to each frozen sample, which was then homogenized with a tissue homogenizer and centrifuged at 3000 rpm at 4°C (Allegra X-12R, Beckman Coulter, Brea, CA). The resulting supernatant was used for the ELISAs and Western blots. The total protein content of each sample was quantified using a commercially available bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL). The interleukin-6 (IL-6) content of each sample was quantified using a commercially available ELISA kit (cat. no. R6000B, R&D Systems, Minneapolis, MN); samples were diluted 1:5 using TPER lysis buffer containing 1% v/v HALT protease inhibitor and the ELISA was performed according to the kit instructions. The IL-1β content of each sample was quantified using a commercially available ELISA kit (cat. no. KRC0011, Invitrogen, Carlsbad, CA); samples were diluted 1:20 using TPER lysis buffer containing 1% v/v HALT protease inhibitor and the ELISA was performed according to the kit instructions. The chemokine (C-X-C motif) ligand 2 (CXCL2) content of each sample was quantified using a commercially available ELISA kit (cat. no. RCN300, R&D Systems); samples were
diluted 1:10 using TPER lysis buffer containing 1% v/v HALT protease inhibitor and the ELISA was performed according to the kit instructions. For each sample, the ELISA result was expressed as the amount of cytokine per mg total protein.

10.2.5. Western blots

For the Western analysis, 20 µl samples were prepared using ~30 µg of each protein sample together with LDS sample buffer and reducing agent (NuPAGE, Invitrogen). The samples were loaded onto NuPage 4-12% Bis-Tris (1.0mm x 10 well) gels (Invitrogen) together with a protein ladder (1:1 of Novex Sharp and Magic Mark protein standards, Invitrogen) and run for 35 min. The protein in each gel was then transferred to a poly(vinyldene difluoride) membrane over 45 min using a Bio-Rad transfer cell (Bio-Rad, Hercules, CA).

The membrane was blocked in a solution of 5% w/v dry milk in TBST buffer (900 ml milliQ water, 100 ml 10x Tris-buffered saline, 1 ml Tween 20) for 1h at room temperature with gentle agitation. After rinsing 3 times with 20 ml TBST (5 min each), the membrane was incubated overnight at 4°C with agitation in primary anti-FcγRIII antibody (H-80, cat. no. sc-20627, Santa Cruz) diluted 1:500 in 5% w/v dry milk in TBST. After equilibrating to room temperature for 1h, the membrane was rinsed 3 times with TBST and then incubated in secondary antibody solution (anti-rabbit HRP (cat. no. PI-1000, Vector, Burlingame, CA) diluted 1:500 in 5% w/v dry milk in TBST) for 2h at room temperature with agitation. After three more rinses in TBST followed by two rinses with milliQ water (20 ml for 2 min each), the membrane was placed in an X-ray cassette, covered with ECL Plus reagent (Amersham, Piscataway, NJ), and exposed to dental X-ray film (BX autoradiography film, MIDSCI, St. Louis, MO). The membrane was
stripped using ReBlot Plus Strong reagent (Millipore, Billerica, MA) for 15 min and the procedure described above was repeated using a primary anti-β-actin antibody (cat. no. 4970, Cell Signaling, Danvers, MA) diluted 1:1000 in 5% w/v dry milk in TBST and the appropriate secondary antibody (cat. no. 7074, Cell Signaling, Danvers, MA) diluted 1:1000 in 5% w/v dry milk in TBST (note: the membrane was incubated in this solution for 1 h instead of 2 h).

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 for minimizing error (320).

10.2.6. Polyplex preparation

The siRNA sequences (siRNA 1 sense: 5'-CCAGCUCUCUAGUGGUUTT-3' and siRNA 2 sense: 5'-GCUCUCUAGUGGUUCCATT-3'; Silencer® siRNA ID nos. 195781 and 195782, respectively; Ambion, Austin, TX) were selected based on a previous study indicating that intra-articular injection of a mixture of siRNA1-PEI and siRNA2-PEI polyplexes significantly reduced inflammation-induced changes for 48 h in the rat TMJ model (307). Each siRNA sequence was reconstituted at a concentration of 3.2 nmol siRNA/μl in nuclease-free water according to the manufacturer’s instructions.

The ratio of PEI to siRNA is commonly expressed as the Nitrogen:Phosphate (N:P) ratio, i.e., the ratio of protonatable amine (N) groups to nucleic acid backbone phosphates (P). The N:P ratio for this study was calculated as previously described (17, 303). To create polyplex-loaded PLGA MPs containing either siRNA1-PEI or siRNA2-
PEI with a 16:1 N:P ratio, 25 µl of the relevant siRNA stock solution was mixed with 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 a few seconds, incubated at room temperature for 20 minutes to allow for polyplex formation, and then immediately used to prepare (siRNA-PEI)-loaded PLGA MPs.

For PLGA MPs 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 Biotechnology, Rockford, IL). Excess salt was removed from the r-PEI solution using dialysis cassettes (cat. no. 66203, Pierce Biotechnology) with a 2000 kDa molecular-weight cutoff. The r-PEI solution was dialyzed against nuclease-free 1× PBS for 1h, then 0.5× PBS for 8h, and then nuclease-free water (for 2 cycles, each 2h). Dialysis was performed at 4°C under dark conditions to prevent quenching of the rhodamine dye. For each cycle, the buffer volume was 200× greater than that of the sample. All materials, including the dialysis cassettes, were treated with RNase Zap (Ambion) to prevent introduction of RNases into the r-PEI solution that might later result in siRNA degradation. Following dialysis, the r-PEI solution was flash-frozen in liquid nitrogen, wrapped in aluminum foil to prevent quenching of the rhodamine, and lyophilized overnight. The r-PEI was then dissolved at 60 mg/ml in nuclease-free water and used to prepare polyplex-loaded MPs. A single r-PEI batch was used for all experiments described herein.

10.2.7. PLGA MP Preparation and Characterization

PLGA MPs were synthesized using an established double emulsion solvent extraction technique (16, 17, 304). 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). To create polyplex-loaded MPs, the aqueous polyplex solution was added to a mixture of PLGA and PEG dissolved in dichloromethane, which was then vortexed to generate the first emulsion, and ultimately added to an aqueous solution containing 0.15% w/v poly(vinyl alcohol) and 1% v/v isopropanol to create the double emulsion. MPs loaded with r-PEI were prepared in aluminum-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.

Each MP formulation contained siRNA-PEI or siRNA-r-PEI polyplexes with a single siRNA sequence (i.e., either siRNA1 or siRNA2). For the in vivo study, the MP formulations containing the two siRNA sequences were mixed in a 50:50 ratio prior to intra-articular injection. However, in vitro release was separately measured from each MP formulation so that the released siRNA and r-PEI could be quantified via comparison to a standard curve derived using polyplexes containing the identical siRNA sequence.

Following synthesis, 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).

**10.2.8. Quantification of Entrapment Efficiency**

The entrapment efficiency of each MP formulation was determined using a previously described protocol (17, 303). Briefly, 15.0 mg of MPs were placed in 0.5 ml dichloromethane and incubated at room temperature until completely dissolved (~20 min). Nuclease-free TE buffer (0.5 ml of 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was added to extract the polyplexes. 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. The extraction was repeated, and the two 0.5 ml aqueous phases were combined and assayed for either siRNA or r-PEI content.

**10.2.9. Polyplex Release**

For each PLGA MP formulation, 15.0 mg samples \((n = 4)\) were suspended in 0.5 ml nuclease-free PBS (pH 7.4) and incubated at 37°C with gentle agitation. At each timepoint (2h, 4h, 8h, 12h, and days 1, 2, 3, 4, 8, 11, 14, 17, 21, 25, 28), samples were collected using an established method (17). The vials were centrifuged at 11,000 × g for 5 min at 4°C, the supernatant was removed and 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 to prevent degradation prior to analysis.
10.2.10. Polyplex Dissociation and Quantification of siRNA Content

The siRNA content of the polyplexes was determined using an established protocol (17). Briefly, 50 μl of each release sample or standard were combined with 100 μl of 0.5% v/v PicoGreen dye (Molecular Probes, Eugene, OR) in alkaline TE buffer (pH 12) and analyzed using a fluorescence plate reader (excitation/emission wavelength = 485/530 nm). The standards consisted of freshly prepared 16:1 N:P ratio polyplexes with the same siRNA sequence (i.e., either siRNA1-PEI or siRNA2-PEI) as the samples. Samples and standards were run in triplicate.

10.2.11. Quantification of r-PEI Content

The release of r-PEI from the PLGA MPs was quantified according to an established protocol (17, 305). Briefly, 100 μl of the standard or sample was added to each well of an opaque 96-well plate and quantified using a fluorescence plate reader (excitation/emission wavelength = 530/620 nm). The standard curve consisted of siRNA-r-PEI polyplexes prepared using the same siRNA sequence as the samples. All samples and standards were run in triplicate.

10.2.12. Dynamic Light Scattering

Dynamic light scattering (DLS) was performed using a 90PLUS particle size analyzer (Brookhaven Instruments, Holtsville, NY) operating at 659 nm wavelength and 37°C. Within the BIC Dynamic Light Scattering software (Brookhaven Instruments) supplied with the DLS apparatus, the accumulated autocorrelation function was analyzed.
via the non-negatively constrained least squares (NNLS) algorithm to derive the intensity-weighted particle size distribution.

10.2.13. Fresh Polyplexes

The DLS results of the release samples were then compared to those of the freshly prepared polyplexes to assist in identifying the polyplex size as previously described (17). For each siRNA type, polyplexes with N:P ratios spanning the range of those observed for the release samples were prepared, incubated at 37°C with gentle agitation, and then their diameter was measured via DLS after 4h and then again after 24h.

10.2.14. Statistics

Meal pattern data are reported as mean ± standard deviation for $n = 9$ rats per group. For each group, meal duration and food intake are expressed as a percentage of the average pre-injection value recorded during the 48 h prior to injection; as previously reported, this method normalizes the data to eliminate inherent differences amongst the groups of rats, while elucidating differences due to the treatments (27). Meal duration and food intake data were analyzed using two-way analysis of variance ($p < 0.05$) to examine differences within each group over time, and also differences amongst groups on each day. Multiple pair-wise comparisons were made using the Bonferroni post-hoc analysis method at 95% confidence.

As previously described, for each sample, the amount of cytokine measured via ELISA was divided by the total amount of protein in that sample. ELISA data are reported as mean ± standard error of the mean for $n = 6$ rats per group. Similarly, the value of each band from the Western blot, as determined via densitometry, was divided
by the value of the corresponding β-actin band. The data are reported as mean ± standard deviation for $n = 6$ rats per group. For both ELISAs and Western analysis, differences amongst groups were determined one-way analysis of variance ($p < 0.05$) followed by Bonferroni post hoc analysis ($p < 0.05$) for multiple comparisons.

MP diameters are presented as mean ± standard deviation ($n = 1500$ particles per MP batch). Entrapment efficiencies are presented as mean ± standard deviation ($n = 3$ samples). For each MP formulation, the release values are presented as a percentage of the total measured amount of siRNA or r-PEI entrapped, determined by extracting the polyplexes from a 15.0 mg sample of the PLGA MPs and quantifying the amount of siRNA or r-PEI present. Data points for cumulative release of siRNA and r-PEI, as well as hydrodynamic diameters of siRNA, PEI, and siRNA-PEI polyplexes, represent mean ± standard deviation for ($n = 4$) samples at each timepoint. Cumulative release and DLS data were analyzed using a repeated measures analysis of variance ($p < 0.05$), followed by Bonferroni post hoc analysis ($p < 0.05$). The diameter of the freshly prepared polyplexes was compared to that of the polyplexes from each release sample with equivalent N:P ratio via analysis of variance ($p < 0.05$), followed by Bonferroni post hoc analysis ($p < 0.05$) for multiple comparisons.

10.3. Results and Discussion

10.3.1. Meal Pattern Analysis

The therapeutic effect of the siRNA-PEI-loaded PLGA MPs was evaluated via computerized meal pattern analysis. This non-invasive technique, whose development
was inspired by the painful, impaired eating in human patients with TMJ disorders, has been used to evaluate the effect of various analgesic agents on TMJ pain (22, 24-27, 307).

The nocturnal meal duration and food intake of all rats in this study are summarized in Figure 10.1. All groups differed significantly \((p < 0.05)\) in terms of meal duration (Figure 10.1a), and all groups except the “CFA only” control and High blank MPs group differed significantly \((p < 0.05)\) in terms of food intake (Figure 10.1b).

Prior to the induction of inflammation (days 2-5), the MP groups did not have any significant irritating effect on either meal parameter. The small dip in food intake seen during days 2-5 for all groups except the “CFA only” control, which did not receive any injection on day 2, was also observed in our previous study of blank PLGA MPs (16) (Figure 8.2), but was not statistically significant \((p > 0.05)\).

The siRNA-PEI-loaded MPs actually consisted of a mixture of two PLGA MP formulations. As detailed in the Methods (page 222), siRNA1-PEI-loaded MPs and siRNA2-PEI-loaded MPs were prepared separately and then mixed in equal parts prior to intra-articular injection. Both siRNA1 and siRNA2 target FcγRIII, and the two sequences only differ by a frame-shift of three nucleotides (see sequences on page 221). The use of siRNA1 together with siRNA2 was motivated by a previous study in the same rat TMJ model, in which injection of a 50:50 mixture of “free” siRNA1-PEI and siRNA2-PEI polyplexes had a significant effect on TMJ inflammation for 48h (307).
Figure 10.1: Impact of siRNA-PEI-loaded MPs on meal parameters

Anti-FcγRIII-siRNA-PEI-loaded PLGA MPs reduced inflammation-induced changes in both (a) meal duration and (b) food intake. Dashed lines indicate the time of MP injection for all groups except “CFA only,” which did not receive MPs. A second dashed line indicates the time that pro-inflammatory CFA was injected. All groups in (a) differ significantly ($p < 0.05$). In (b), groups marked with * differ significantly from all other
groups, while groups marked with ** differ significantly from all other groups but not each other \( (p < 0.05) \). 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.

Following CFA injection, siRNA-PEI-loaded PLGA MPs significantly \( (p < 0.05) \) reduced inflammation-induced changes in meal duration (Figure 10.1a), with the Low anti-FcgR3 siRNA MPs group having the most therapeutic effect. At the same time, siRNA-PEI-loaded MPs significantly increased the rate at which food intake returned to baseline \( (p < 0.05) \), with the High anti-FcgR3 siRNA MPs group having the most therapeutic effect.

During that same time, the High blank MPs group increased meal duration compared to the “CFA only” control \( (p < 0.05) \), and the “Low blank MPs” group decreased food intake more than the CFA only control \( (p < 0.05) \). This indicates that the presence of MPs together with CFA had an additive irritating effect on the TMJ. However, this irritation, as well as some of the irritation induced by the CFA itself, were treated by the release of siRNA-PEI polyplexes, as evidenced by the significantly lower meal duration \( (p < 0.05) \) and significantly higher food intake \( (p < 0.05) \) of the polyplex-loaded MP groups after day 5 (Figure 10.1).

The magnitude of these therapeutic effects at day 9 is comparable to previously reported results (307) observed just one day after injection of a solution of free anti-FcγRIII-siRNA-PEI polyplexes. However, in that case, 11 μg of siRNA were delivered per joint, while only 6-9 μg siRNA/joint were released from the MPs over the course of
this entire study. This suggests that our intra-articular PLGA MP-based system is a more efficient means of delivering siRNA-PEI polyplexes to the TMJ.

10.3.2. Inflammatory Cytokine Levels

FcγRIII is a cell-surface receptor on macrophages and neutrophils whose presence stimulates the secretion of several inflammatory cytokines (307). Figure 10.2 shows the protein levels of two such inflammatory cytokines, IL-6 and IL-1β, as well as the level of chemokine (C-X-C motif) ligand 2 (CXCL2), a macrophage product expressed at sites of inflammation. Levels of all cytokines were measured via ELISA using TMJ retrodiscal tissue harvested on the final day of the experiment.

Anti-FcγRIII-siRNA-PEI-loaded PLGA MPs significantly ($p < 0.05$) reduced IL-6 levels compared to all other groups. The Low anti-FcγR3 siRNA MPs also significantly reduced IL-1β levels ($p < 0.05$), a finding consistent with the meal duration findings (Figure 10.1a), where this group had a greater therapeutic effect than the higher dose of siRNA-PEI-loaded MPs. Also consistent with the meal duration data, the High blank MPs groups had slightly higher ($p < 0.05$) IL-6 and CXCL2 levels than all other groups, including the CFA only control.
Figure 10.2: Effect of siRNA-PEI-loaded MPs on cytokine levels

Anti-FcγRIII-siRNA-PEI-loaded PLGA MPs reduced protein levels of two inflammatory cytokines: (a) interleukin-6 (IL-6) and (b) IL-1β, and did not affect levels of (c) chemokine (C-X-C motif) ligand 2 (CXCL2). ELISAs were performed using protein extracted from the retrodiscal TMJ tissue on day 9 of the experiment. 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. 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 \((p < 0.05)\).

The level of IL-1\( \beta \) in the inflamed rat TMJ has been previously reported 48h after injection of the same CFA dose (15 \( \mu \)g/joint) used in this study (~900 pg IL-1\( \beta \)/mg total protein) (307), as well as 7 days after injection of a much higher (30 \( \mu \)g/joint) CFA dose (~1000 pg IL-1\( \beta \)/mg total protein) (321). In this latter study, IL-6 levels in the inflamed TMJ were also reported at the 7 day timepoint (~50 pg IL-6/mg total protein) (321). Compared to these published findings, IL-6 levels in this study (Figure 10.2a) were much higher for all groups, while IL-1\( \beta \) levels were (Figure 10.2b) were much lower. In both cases, the cytokine levels exceeded the reported baseline values in the non-inflamed rat TMJ, which are ~20 pg IL-6/mg total protein and ~0 pg IL-1\( \beta \)/mg total protein (321), indicating that TMJ inflammation had not yet subsided.

### 10.3.3. Fc\( \gamma \)RIII Expression

The mechanism of the anti-Fc\( \gamma \)RIII siRNA delivered in this study, as with all siRNA molecules, is to silence a specific gene by binding to messenger RNA and blocking protein translation (272, 273). To correlate the therapeutic effects observed via meal pattern analysis with the known mechanism of siRNA, the TMJ tissue expression of Fc\( \gamma \)RIII (Figure 10.3) was measured via Western blot densitometry using TMJ retrodiscal tissue harvested on the final day of the experiment. Although the PLGA MPs are capable of delivering siRNA-PEI polyplexes for 28 days (see release Results below), day 9 was selected as the endpoint for the study by taking into consideration the established
duration of CFA-induced inflammation in the rat model (22). Specifically, the study ended prior to the complete resolution of TMJ inflammation, so that differences in protein expression of inflammatory cytokines and FcγRIII might still be present.

Figure 10.3: Effect of anti-FcγRIII-siRNA-PEI-loaded MPs on FcγRIII expression
To evaluate the therapeutic mechanism of anti-FcγRIII-siRNA-PEI-loaded PLGA MPs, the tissue expression of the siRNA target, FcγRIII, was measured via densitometry of Western blots using protein extracted from the retrodiscal TMJ tissue on day 9 of the study. The groups are in the same order and represented by the same patterns as in all previous figures (from left to right: High siRNA-PEI loaded MPs, Low siRNA-PEI-loaded MPs, High blank MPs, Low blank MPs, and CFA only). Values for each group are expressed relative to the value of the β-actin loading control, also measured via Western blot densitometry. The value for the High Blank group was set as 100%. Data points represent the mean ± standard deviation for \( n = 6 \) rats. The group marked with * differs significantly from all other groups \( (p < 0.05) \).

The FcγRIII expression (Figure 10.3) mirrored the IL-6 and CXCL2 levels (Figure 10.2), as well as the meal duration data (Figure 10.1a). 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 ($p > 0.05$). These findings for day 9 of our study are qualitatively consistent with a previous study in which anti-FcγRIII-siRNA-PEI polyplexes were injected into the same inflamed rat TMJ model (307). 48h after CFA injection, the siRNA treated rats had lighter FcγRIII bands on the Western blot compared to CFA-only controls. However, densitometry data were not reported in that study (307).

Qualitative analysis of immunostaining for FcγRIII was also consistent with the Western blot findings. Figure 10.4 depicts images from a rat in the High anti-FcgR3 siRNA MPs group.

![CD16 (target of siRNA) Overlap CD14 (positive control)](image)

**Figure 10.4: Immunostaining for FcγRIII expression with siRNA-PEI-loaded MPs**

A representative image of FcγRIII (i.e., CD16) expression with anti-FcγRIII-siRNA-PEI-loaded PLGA MPs. The positive control (CD14) is present on inflammatory cells that would normally also express the siRNA target, FcγRIII (CD16). CD16-positive cells were localized near blood vessels, suggesting that they were newly arrived to the site of the inflammation. Most of the cells in the tissue did not express CD16, as expected. The scale bar indicates 50 μm and applies to all panels.

The positive control (CD14) is a marker present on the same inflammatory cell types that would normally express the siRNA target, FcγRIII (CD16) (307). However,
cells expressing the siRNA target (FcγRIII/CD16) were mainly seen within and near
blood vessels, suggesting that they were newly arrived to the site of the inflammation.
Most of the cells in the tissue of rats from the polyplex-treated groups did not express the
siRNA target, as expected.

10.3.4. Entrapment efficiency of siRNA and PEI in the microparticles

The entrapment efficiency of siRNA and PEI within the PLGA MPs is
summarized in Table 10.1, along with the diameter of the PLGA MPs. There were no
statistically significant differences amongst the groups ($p > 0.05$).

| Table 10.1: Entrapment efficiency of siRNA and PEI in the PLGA MPs |
|---------------------|---------------------|---------------------|---------------------|
| MP contents         | PLGA MP yield (%)$^a$ | Theoretical loading (µg / mg PLGA MPs)$^b$ | Entrapment efficiency (% of theoretical loading)$^c$ | MP diameter (µm)$^d$ |
| siRNA1-PEI polyplexes | 64 ± 5              | siRNA: 7.4 ± 0.5    | siRNA: 85 ± 12      | 18 ± 1             |
|                     |                     | PEI: 11 ± 1        | PEI: 76 ± 7         |                    |
| siRNA2-PEI polyplexes | 68 ± 5              | siRNA: 6.1 ± 0.3   | siRNA: 91 ± 11      | 18 ± 1             |
|                     |                     | PEI: 13 ± 1        | PEI: 78 ± 5         |                    |
| Blank               | 83 ± 2              | none               | none                | 15 ± 2             |

$^a$ Final weight of PLGA MPs as a percentage of the starting amount of PLGA and PEG (248 mg per batch). Values represented as 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%
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.

10.3.5. Release of siRNA and PEI from PLGA MPs

Figure 10.5 illustrates the cumulative release of siRNA and r-PEI from the MP formulations. Although MP formulations containing siRNA1-PEI and siRNA2-PEI polyplexes were mixed in a 50:50 ratio prior to intra-articular injection, the MPs were maintained separate for the \textit{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.

The release profiles of the two siRNA sequences were statistically equivalent \((p > 0.05)\). The release followed a triphasic pattern consisting of an initial burst release for 24h, followed by a lag period, after which release resumed around day 8. Approximately 60-65% of the encapsulated siRNA was released during the burst period.

The PEI release profiles of the two MP formulations completely overlap in Figure 10.5b and were also statistically equivalent \((p > 0.05)\). The PEI release followed a biphasic pattern consisting of an initial burst release for 24h, in which ~20% of the encapsulated PEI was released, 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.
PLGA MP formulations encapsulating polyplexes consisting of the two different siRNA sequences had statistically equivalent (a) siRNA release profiles \((p > 0.05)\), 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, approximately linear PEI release until day 28. Error bars are included for all data points, though they are too small to resolve. Two sets of MPs were prepared for each polyplex-loaded formulation because quantification of siRNA and PEI content and release required the use of fluorometric assays that had overlapping emission and excitation wavelengths. MPs for siRNA measurements were loaded with siRNA-PEI polyplexes, while MPs for PEI measurements were loaded with polyplexes containing rhodamine-labeled PEI. Values for each group are expressed as a percentage of the amount of siRNA or PEI initially encapsulated (= entrapment efficiency \(\times\) theoretical loading from Table 10.1). Data points represent the mean \(\pm\) standard deviation for \(n = 4\) samples, each consisting of 15 mg of MPs suspended in 0.5 ml PBS and incubated at 37°C with gentle agitation.

At each timepoint, the siRNA and r-PEI release data were used to calculate the N:P ratio, as summarized in Table 10.2 ("N" values reported after setting "P" to 1 for each calculation). A repeated measures analysis of the calculated N:P ratios indicated that
the N:P ratios of the polyplexes released from the two MP formulations did not differ over time ($p > 0.05$). However, the calculated N:P ratios significantly differed ($p < 0.05$) from the ideal N:P value of 16:1 at every timepoint.

### Table 10.2: Calculated N:P ratios over time

<table>
<thead>
<tr>
<th>Day</th>
<th>siRNA1-PEI-loaded PLGA MPs</th>
<th>siRNA1-PEI-loaded PLGA MPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>2 ± 0.2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>3 ± 1&lt;sup&gt;c&lt;/sup&gt;</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 ± 0.2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>0.4 ± 0.1</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>7 ± 1</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>4</td>
<td>14 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>8</td>
<td>38 ± 8</td>
<td>34 ± 7</td>
</tr>
<tr>
<td>11</td>
<td>2 ± 1</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>14</td>
<td>0.4 ± 0.2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>17</td>
<td>0.3 ± 0.2</td>
<td>1 ± 0.4</td>
</tr>
<tr>
<td>21</td>
<td>1 ± 0.3</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>25</td>
<td>0.1 ± 0.0</td>
<td>1 ± 1</td>
</tr>
<tr>
<td>28</td>
<td>0.1 ± 0.0</td>
<td>0.3 ± 0.1</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 (17). 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 ($C_2H_5N$) of linear PEI.
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.

10.3.6. Size of siRNA-PEI polyplexes released from PLGA MPs

The polyplexes released from siRNA1-PEI-loaded MPs over 28 days were significantly larger than those released from siRNA2-PEI-loaded MPs (135 ± 50 nm vs. 117 ± 50 nm diameter; p < 0.05) (Figure 10.6b). The peaks in the 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 10.6a. The orange arrows indicate peaks that were statistically equivalent to freshly prepared polyplexes (p > 0.05). The green arrows indicate peaks that were statistically equivalent to pure PEI (p > 0.05). The yellow arrows indicate peaks that were statistically different from pure polyplexes with the same N:P ratio (p < 0.05). To account for larger peaks that might arise from PLGA MPs and their derivatives, the particles present in the release medium of non-drug loaded MPs was also characterized via DLS (gray shading in Figure 10.5).
Shading indicates overlap with peaks from empty PLGA MPs.
Figure 10.6: Size of polyplexes released from PLGA MPs over time

Representative DLS curves at 37°C are shown in (a) for each formulation at each timepoint. Samples released from siRNA-PEI polyplex-loaded PLGA MPs typically contained one population of particles that were ~150 nm in diameter, as shown in (b). Specifically, siRNA1-PEI polyplexes had an average diameter of 135 ± 50 nm, while siRNA2-PEI polyplexes had an average 117 ± 50 nm diameter. The green arrows in (a) indicate peaks that were statistically equivalent in size to pure PEI. The orange arrows in (a) indicate peaks that were statistically equivalent to freshly prepared polyplexes of the same N:P ratio. The yellow arrows in (a) 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. The white-filled data points in (b) correspond to groups also shown in (a) where there were no detectable polyplexes (e.g., only free PEI was detected).
Unlike our previous study of siRNA-PEI-loaded PLGA MPs (17), in which free PEI was almost never observed in the release medium, several timepoints contained identifiable free PEI peaks (green arrows in Figure 10.5). This likely reflects the large increase in the amount of PEI loaded into the MPs in this study compared with the previous study. In the previous study, the absence of free PEI in the DLS samples was presumed to reflect the binding of the cationic PEI with anionic PLGA MP derivatives.

However in this study, the greatly increased amount of PEI may have outweighed the number of anionic molecules present. This might also explain the superior therapeutic effect of the low dose of siRNA-PEI-loaded MPs compared to the higher dose. In addition to the MPs, which might act as an irritant in the setting of TMJ inflammation, the additional PEI released by the higher dose of siRNA-PEI MPs might also counteract the anti-inflammatory effect of the siRNA, since free PEI is highly cytotoxic (295, 310, 315, 316). However, it is important to note that in a recent study involving the intra-articular injection of a much larger dose of siRNA-PEI polyplexes into the rat TMJ, no adverse effect was seen compared to injection of plain siRNA (307). In fact, superior reduction of inflammation-induced changes to meal duration was observed with polyplex delivery (307).

The observed diameters of the siRNA-PEI polyplexes released from PLGA MPs (Figure 10.6b) were below the established threshold (~150 nm) for intracellular uptake of siRNA-PEI polyplexes and subsequent gene silencing (296, 316). This is consistent with the observed in vivo efficacy of the siRNA-PEI-loaded PLGA MPs in alleviating TMJ inflammation.
10.4. 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. To our knowledge, this is the first intra-articular controlled release system for the TMJ. The PLGA MP formulations showed sustained release of siRNA and PEI over 28 days in vitro. In a week-long in vivo study in a rat TMJ model, the MPs reduced inflammation-induced changes in meal parameters and inflammatory cytokine expression. This novel intra-articular controlled release system not only represents a great improvement to TMJ therapeutics, but has the potential to be adapted and used to mitigate tissue damage and pain in rheumatoid arthritis and various other inflammatory conditions.
References


126. de Castro, L.F., Lozano, D., Dapia, S., Portal-Nunez, S., Caeiro, J.R., Gomez-Barrena, E., and Esbrit, P. Role of the N- and C-terminal fragments of parathyroid-


for the management of rheumatoid arthritis with biological disease-modifying antirheumatic drugs: a systematic literature review informing the EULAR recommendations for the management of RA. Ann Rheum Dis 69, 976, 2010.


\alpha\] blockers: perioperative interruption of tumour necrosis factor-\[
\alpha\] blockers decreases complications? Rheumatology 49, 341, 2010.


