Pharm Res (2011) 28:1370–1384
DOI 10.1007/s11095-010-0354-9

RESEARCH PAPER

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

Paschalia M. Mountziaris • David C. Sing • Sue Anne Chew • Stephanie N. Tzouanas • E. Dennis Lehman • F. Kurtis Kasper • Antonios G. Mikos

Received: 1 November 2010 / Accepted: 14 December 2010 / Published online: 24 December 2010

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 500–1,000 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.

P. M. Mountziaris • D. C. Sing • S. A. Chew • S. N. Tzouanas • E. D. Lehman • F. K. Kasper • A. G. Mikos (✉)
Department of Bioengineering, Rice University
P.O. Box 1892, MS 142
Houston, Texas 77251-1892, USA
e-mail: mikos@rice.edu

KEY WORDS intra-articular · microparticle · polyplex · temporomandibular joint

ABBREVIATIONS

anti-TNF-α siRNA	siRNA targeting TNF-α
DLS	dynamic light scattering
MPs	microparticles
N:P ratio	Nitrogen:Phosphate ratio
PBS	phosphate-buffered saline
PEG	poly(ethylene glycol)
PEI	poly(ethylenimine)
PLA	poly(DL-lactic acid)
PLGA	poly(DL-lactic-co-glycolic acid)
r-PEI	rhodamine-conjugated PEI
siRNA	small interfering RNA
TMJ	temporomandibular joint
TNF-α	tumor necrosis factor-α

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 (1). In severe TMJ degeneration, pain can no longer be treated with oral medication, due to the risk of systemic toxicity; 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 (1,2).

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 (3,4). 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 3 weeks) and electroporation of the affected joints to induce intracellular uptake of the charged siRNA and achieve long-term treatment of joint inflammation (5,6). 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 (7,8).

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 (9). 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 (10,11) and nanoparticles (12–15) 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 (8). 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.

MATERIALS AND METHODS

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 four distinct siRNA-PEI-loaded PLGA MP formulations, as summarized in Tables I, II. For siRNA release, control groups consisted of siRNA-only-loaded PLGA MPs with equivalent "high" or "low" siRNA loading (Table I) 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 II). 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.

Polyplex Preparation

The siRNA sequence (sense: 5′-CCCACGUCGUAGC AAACCTT-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* (5). 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 min 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 (16). 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.

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 4,600; 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 42,500±1,600 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)

Table I Entrapment Efficiency of siRNA in the PLGA Microparticles

siRNA loading	N:P ratio	PLGA MP yield (%)[a]	Theoretical siRNA loading (μg siRNA/mg PLGA MPs)[b]	Entrapment efficiency (% of theoretical loading)[c]	MP diameter (μm)[d]
Low	16:1	81 ± 3	0.067 ± 0.002	77 ± 8	31 ± 2
	8:1	88 ± 3	0.062 ± 0.002	61 ± 13	28 ± 4
	No PEI	68 ± 1	0.080 ± 0.001	55 ± 5[e]	27 ± 5
High	16:1	76 ± 4	0.36 ± 0.02	20 ± 4[f]	31 ± 2
	8:1	85 ± 2	0.32 ± 0.01	27 ± 5[f]	32 ± 2
	No PEI	72 ± 4	0.37 ± 0.01	39 ± 4[g]	26 ± 8

[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 = 1,500 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 ($p < 0.05$)

using a polystyrene standard curve (Fluka, Switzerland). MPs were synthesized using a previously described double-emulsion solvent extraction technique (9,17,18). 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 48 h, 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).

Quantification of Entrapment Efficiency

The entrapment efficiency of each MP batch was determined using a previously described protocol for siRNA-loaded polymeric MPs (16). Briefly, 15.0 mg of MPs were placed in 0.5 ml dichloromethane and incubated at room temperature until completely dissolved (1-2 h). 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

Table II Entrapment Efficiency of PEI in the PLGA Microparticles

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

[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 = 1,500 microparticles per batch)

[e] Indicates values that do not differ from each other but differ from all other entrapment efficiencies ($p < 0.05$)

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.

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 (2 h, 4 h, 8 h, 12 h, 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.

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 (19), 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.

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 (20). Briefly, 50 µl of each release sample or standard were added to the wells of an opaque 96-well plate. One-hundred µl of 0.5 v/v% PicoGreen dye (Molecular Probes, Eugene, OR) in alkaline TE buffer (pH 12, increased from pH 7.4 using 1 N 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)).

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.

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 4 h and then again after 24 h. The N:P ratios (listed in Fig. 3) and incubation times (4 h and 24 h) of the freshly prepared polyplexes were selected to enable direct comparison with polyplexes released from PLGA MPs (Table III).

Statistics

MP diameters are presented as mean±standard deviation ($n=1{,}500$ particles per MP formulation). Entrapment efficiencies are presented as mean±standard deviation

Table III Calculated N:P Ratios over Time

Day	Low siRNA		High siRNA	
	16:1 N:P[a]	8:1 N:P	16:1 N:P	8:1 N:P
0.1	11 ± 2[b]	4 ± 1[c]	19 ± 3	5 ± 1[c]
0.2	10 ± 5	7 ± 2	49 ± 9[d]	12 ± 4
0.3	3 ± 1[d]	15 ± 3[c]	107 ± 19[d]	55 ± 14[c]
0.5	4 ± 2[d]	1 ± 1[c]	73 ± 36	8 ± 4
1	7 ± 5[d]	13 ± 4	73 ± 42	10 ± 4
2	14 ± 5	14 ± 4	13 ± 5	4 ± 1[c]
3	244 ± 155	104 ± 61	15 ± 5	17 ± 9
4	243 ± 150	581 ± 500[e]	37 ± 15	7 ± 4
8	33 ± 15	N/A[f]	17 ± 8	7 ± 3
12	26 ± 12	24 ± 20	13 ± 6	4 ± 2
16	4 ± 2[d]	5 ± 3	2 ± 1[d]	5 ± 1
19	2 ± 1[d]	4 ± 2[c]	1 ± 0.4[d]	4 ± 1[c]
22	7 ± 4[d]	1 ± 0.5[c]	5 ± 2[d]	2 ± 0.5[c]
25	1 ± 0.4[d]	5 ± 2[c]	2 ± 1[d]	3 ± 1[c]
28	1 ± 0.3[d]	5 ± 2[c]	1 ± 0.3[d]	4 ± 1[c]

[a] N:P ratios expressed with P set to 1, e.g., an N:P of 11:1 is expressed as "11"

[b] 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.

[c] Value differs significantly from ideal N:P ratio of 8:1 ($p < 0.05$)

[d] Value differs significantly from ideal N:P ratio of 16:1 ($p < 0.05$)

[e] 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.

[f] 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.

($n = 3$ 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 (4 h and 24 h) 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.

RESULTS

Microparticle Diameter

The MPs had a diameter of 26–34 μm, and none of the formulations differed significantly from each other in size ($p > 0.05$) (Tables I, II).

Entrapment Efficiency of siRNA and PEI in the Microparticles

The entrapment efficiency of siRNA (Table I) differed significantly ($p < 0.05$) amongst some of the PLGA MP formulations. Specifically, MPs with low siRNA theoretical loading (~0.07 μg siRNA/mg PLGA MPs) had significantly higher siRNA entrapment efficiencies (77 ± 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 I). 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 II).

Release of siRNA from PLGA MPs

Each MP formulation had a unique siRNA cumulative release profile ($p < 0.05$) over 28 days (Fig. 1a). The release followed a triphasic pattern consisting of an initial burst release for 24 h, 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 24 h burst release from the "low siRNA only" MPs (29 ± 1%)

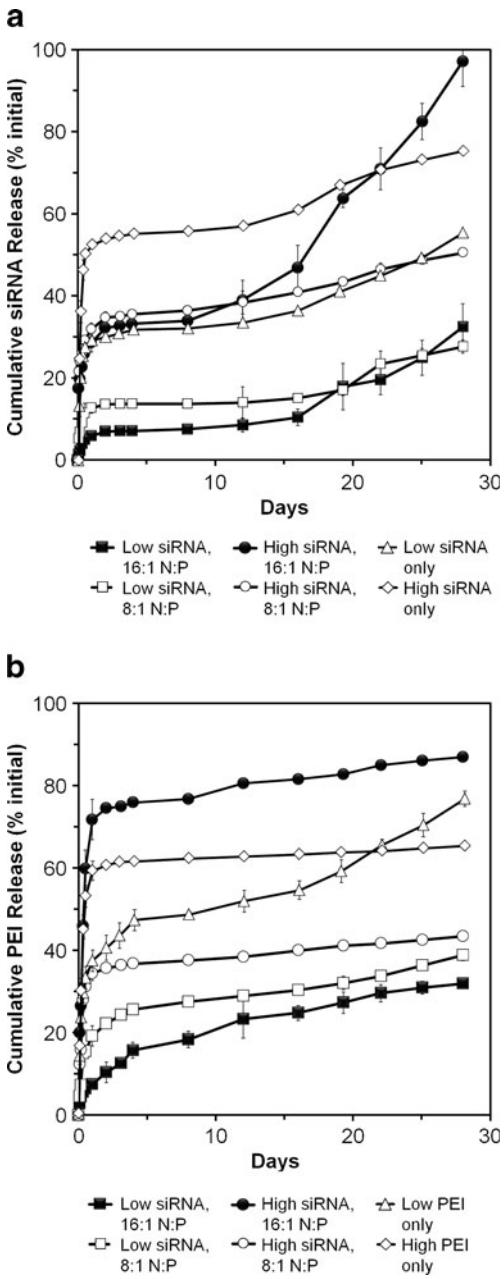

Fig. 1 All PLGA MP formulations had unique (**a**) siRNA and (**b**) PEI cumulative release profiles ($p < 0.05$). Cumulative siRNA release (**a**) followed a triphasic pattern consisting of an initial burst release for 24 h, followed by a lag period, after which release resumed around day 12. The PEI profiles (**b**) also had a 24 h burst release period, followed by gradual, approximately linear PEI release until day 28. The four polyplex-loaded MP formulations and the two siRNA-only or PEI-only groups all had significantly different cumulative release profiles ($p < 0.05$). 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; 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 Tables I, II). 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.

significantly exceeded ($p < 0.05$) that of corresponding "low siRNA" formulations loaded with siRNA-PEI polyplexes (Fig. 1a).

Main effects analysis (Fig. 2a) 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.

Release of PEI from PLGA MPs

For each polyplex-loaded formulation, the PEI and siRNA profiles differed. The PEI profiles had a 24 h burst release period that was followed by gradual, approximately linear PEI release until day 28 (Fig. 1b). The four polyplex-loaded MP formulations and the two PEI-only groups all had significantly different cumulative release profiles ($p < 0.05$) (Fig. 1b). Burst release values ranged from 8 ± 1% to 72 ± 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 II), the vast majority of PEI release occurred during the burst period (at least 80 ± 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 (Fig. 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 μg siRNA/mg PLGA MPs ($p < 0.05$).

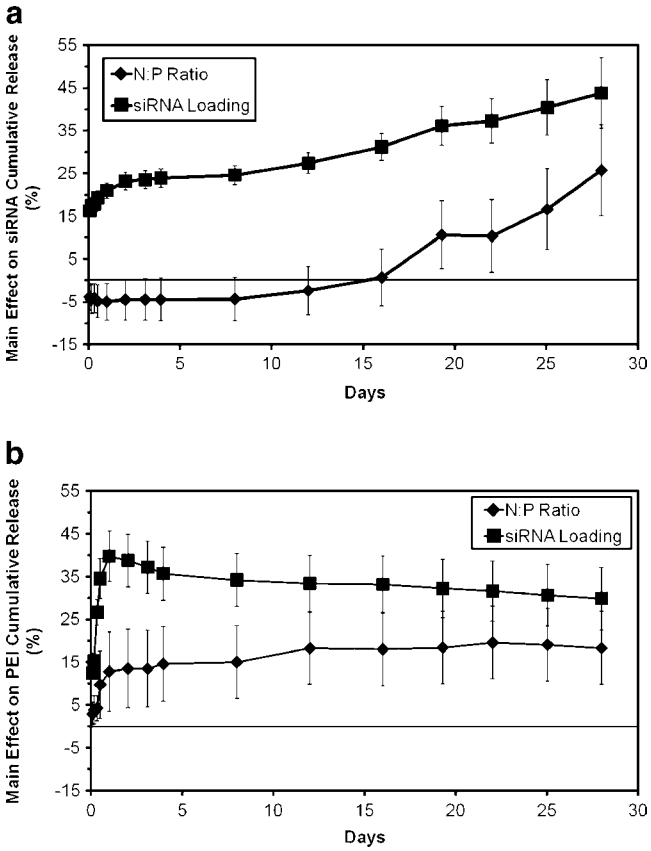

Fig. 2 Main effects analysis of the polyplex-loaded PLGA MPs indicated that siRNA loading and the N:P ratio affected release of both (**a**) siRNA and (**b**) PEI. The vertical axis represents the change in cumulative (**a**) siRNA or (**b**) PEI release that results when either of the two factors (N:P ratio or siRNA loading) is changed from the low to the high value. Increasing siRNA loading from ~0.07 to ~0.35 μg siRNA/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 (2 h 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.

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 III; "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 III). 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 2 h (0.1 days) and significantly greater than 8:1 ($p < 0.05$) at 8 h (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 8 h (0.3 days), 12 h (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 4 h (0.2 days) and 8 h (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 III).

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 (Fig. 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 ($p > 0.05$) (Fig. 3b), all of the freshly prepared polyplex samples had a population of particles in the 50–250 nm range (Fig. 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 4 h to 24 h (Fig. 3b, middle panel), resulting in the release of free PEI (Fig. 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 (Fig. 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 (Fig. 3a, third row *vs.* bottom row).

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 (Fig. 4a), consistent in size with the freshly-prepared siRNA-PEI polyplexes (Fig. 3b, middle panel). The polyplexes released from PLGA MPs showed little variation in diameter despite large variations in N:P ratio (Table III). A repeated measures analysis of the average polyplex

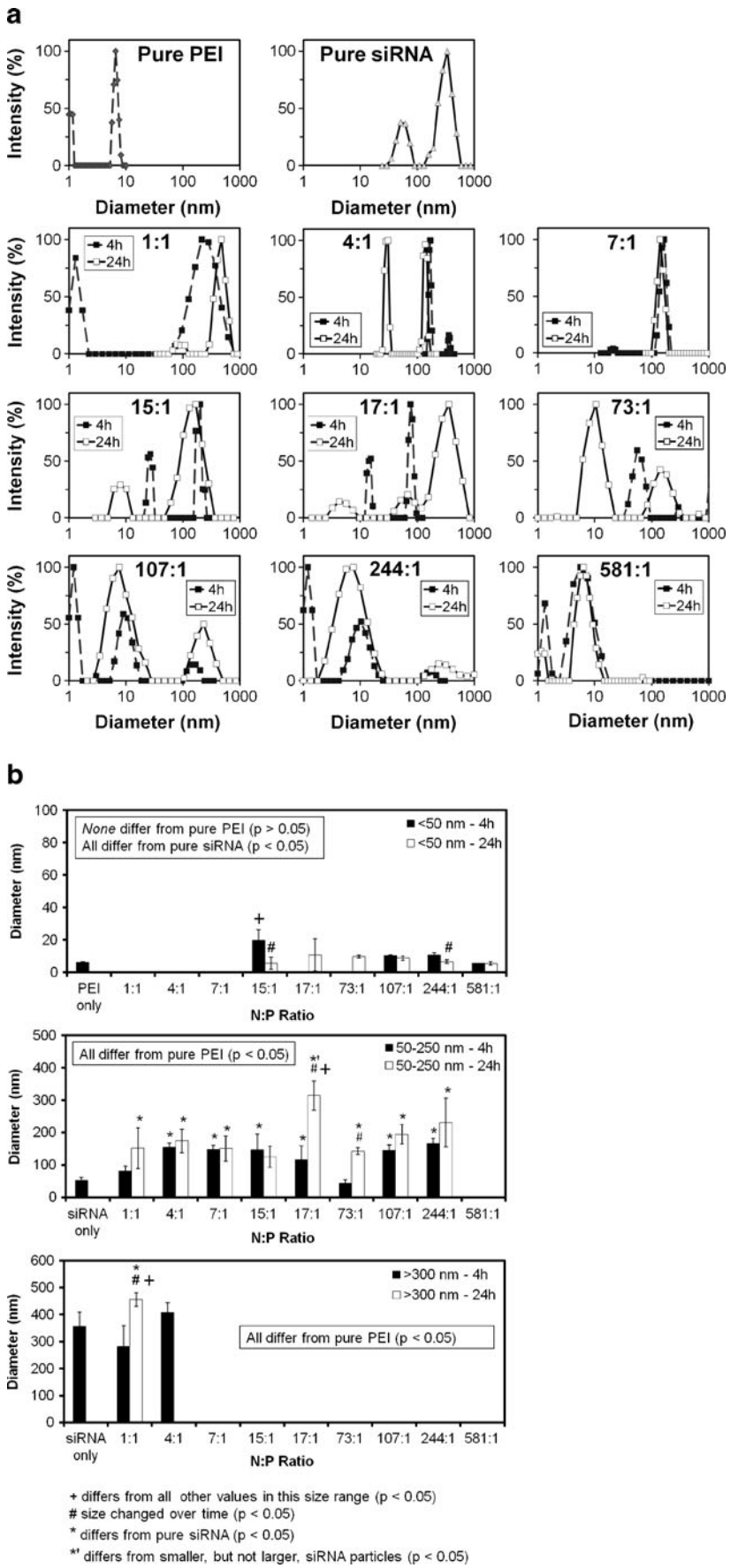

Fig. 3 Freshly prepared polyplexes with varying N:P ratios (from 1:1 to 581:1) had similar hydrodynamic diameters via DLS after 4 h and 24 h incubation at 37°C. **a** Many samples contained more than one population of particles (multiple peaks). **b** The diameters were separated into three ranges: <50 nm, 50–250 nm, or >300 nm, 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 24 h compared to 4 h. 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**); the bar charts in (**b**) summarize the average hydrodynamic diameter ± standard deviation for all ($n = 3$) samples.

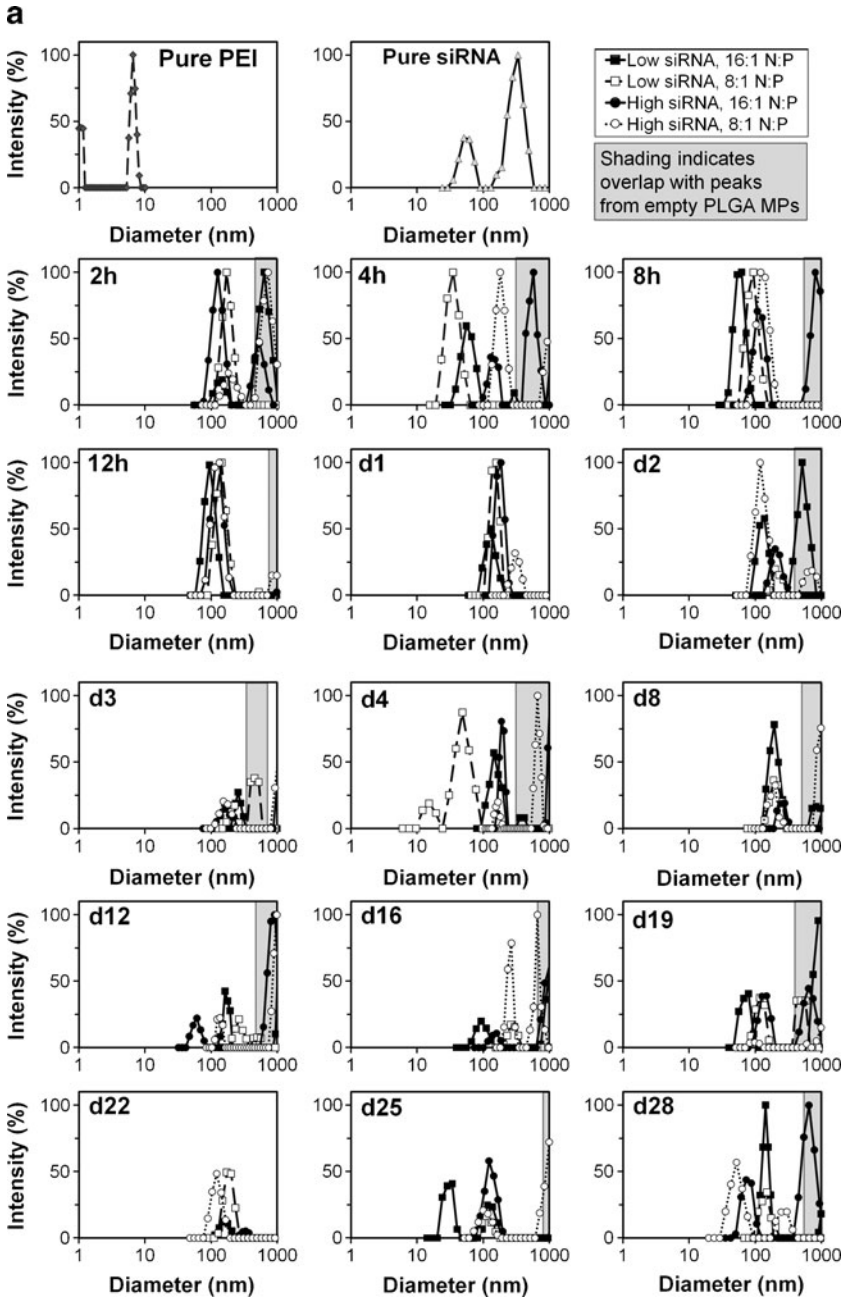

Fig. 4 Samples released from siRNA-PEI polyplex-loaded PLGA MPs contained more than one population of particles (multiple peaks) as detected via DLS (**a**). All samples contained a population of particles that were <300 nm in size. In most cases, the smallest particles were ~150 nm in diameter and were 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; 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.

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") (Fig. 4b). In addition to the polyplexes, many formulations contained a variety of larger (500–1,000 nm) particles at various timepoints; in most cases, the diameter

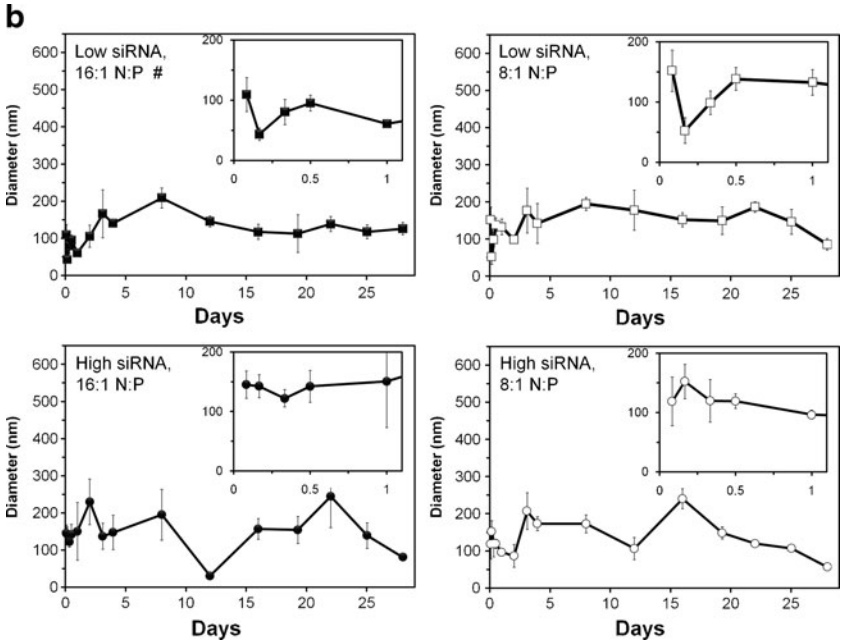

Fig. 4 (continued)

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 Fig. 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 4 h in Fig. 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 ± 10 nm) free siRNA particles.

The released siRNA-PEI polyplexes (Fig. 4) were similar in size to the freshly prepared samples with equivalent N:P ratios (Fig. 3), with a few exceptions. Differences were mainly observed in samples from the burst release period. At the 12 h timepoint (Fig. 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 4 h 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 III) contained 140 ± 50 nm diameter particles, much larger than the freshly prepared 581:1 N:P samples after either 4 h or 24 h (Fig. 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 III), as well as the "low siRNA, 8:1 N:P" 4 h 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" 12 h release samples presented a unique case: the 95 ± 13 nm particles detected (Fig. 4b) were significantly smaller ($p<0.05$) than fresh polyplexes of equivalent 4:1 N:P ratio, but larger than free siRNA ($p<0.05$).

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 (7). In addition to protecting siRNA from degradation by ubiquitous extracellular nucleases and promoting cellular uptake of released siRNA (8), we anticipate that PEI will eliminate the need for joint electroporation (5,6) to achieve transfection. In a recent short-term study, a single intra-articular injection of siRNA-PEI effectively treated painful TMJ

inflammation for 48 h in our intended rat model. The anti-inflammatory siRNA was internalized within cells of the rat TMJ tissue without the need for electroporation (21). 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 (12), while another reported that siRNA-PEI-loaded PLGA MPs achieved superior *in vivo* gene silencing compared to siRNA-only-loaded MPs (11). In the present work, PEI modulated siRNA release from PLGA MPs (Fig. 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 (Fig. 1a), which had the highest PEI loading (Table II).

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") (Tables I, II). 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 (9,18), 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) (9,18). 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 II) than those with unlabeled PEI (Table I). 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) (Tables I, II). 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 (Tables I, II) 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 II) 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 ($p>0.05$; Tables I, II), 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 (9). In that study, the maximal amount of blank PLGA MPs that could be suspended and reproducibly injected was ~2.5 mg per TMJ (9). To achieve an equivalent amount of siRNA to that injected as a free drug solution in the *in vivo* rodent knee electroporation studies (5,6) and in the recent TMJ study (21), PLGA MP loading would need to be one order of magnitude higher than that of the "high siRNA" PLGA MP formulations (Table I). However, *in vivo* efficacy of siRNA-PEI-loaded PLGA MPs has been reported for MPs with much lower loading (11), 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 I) 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) (10,11), the total amount of MPs used to achieve *in vivo* efficacy was much higher (~10 mg) (11) 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 (16). Injection of 3 mg of PLA MPs (loaded with 0.2 μg siRNA/mg MPs) effectively treated an *in vivo* model of peritoneal inflammation (16). The loading value used for the PLA MPs falling

within the range (0.2–0.4 μg siRNA/mg PLGA) reported for siRNA-loaded PLGA nanoparticles (12,15). Consequently, we chose the upper limit of this range as the higher siRNA loading value (~0.35 μg siRNA/mg PLGA MPs; Table I) 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 (22), 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 (Fig. 1a) was released by day 28. Since the detection limit of the PicoGreen dye (20 base pairs (23)) 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 (11,14).

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; thus, PEI entirely blocks binding of fluorescent siRNA-binding dyes (PicoGreen, ethidium bromide, *etc.*) (8,24). Several studies have reported the use of fluorescent siRNA-binding dyes to quantify siRNA content in siRNA-PEI polyplexes (16,25), 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 (11), 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 (26).

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 (20). Since RNA is more susceptible to base-catalyzed degradation than DNA (8,27), 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 (5,000 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 (20)). 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 (26). However, dissociation of siRNA-PEI polyplexes with higher N:P ratios was not described in that study (26).

The 8:1 and 16:1 N:P ratios used for the various PLGA MP formulations (Table I) 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 (11,13,16,21,24). A recent study of PLA MPs encapsulating polyplexes consisting of murine anti-TNF-α siRNA and 25 kDa branched PEI (16) 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 I, and 8:1 N:P polyplexes released from the PLA MPs effectively treated an *in vivo* model of peritoneal inflammation (16). Since the efficacy of gene silencing varies according to siRNA sequence and target cell type (28), 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) (27). Dissociation of the polyplexes enabled measurement of the cumulative release of each component (Fig. 1), which in turn enabled calculation of the approximate N:P ratio at each timepoint (Table III; "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 (Fig. 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 (Fig. 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 24 h 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 II), resulted in increased PEI burst release (Fig. 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 (19). 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 (19). 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 (Fig. 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 III) 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 (Fig. 1a) occurring at the same time as continued, nearly linear PEI release (Fig. 1b). Although the calculated N:P ratio varied greatly, from 1:1 to 581:1 (Table III), 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 (Fig. 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 III); the "low siRNA, 8:1 N:P" sample from day 4 had a small free-PEI peak (Fig. 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 (11). This sequestration of PEI is beneficial, since cytotoxicity is a major limitation of PEI as a siRNA transfection vehicle (7,24,29,30). 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 (29). 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 siRNA alone (21).

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; Fig. 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 (Figs. 3a, 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 (8). The presence of pure siRNA aggregates (>300 nm particles; Fig. 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) (Fig. 3b) are consistent with a phenomenon that has been recently described in detail via DLS analysis of plasmid DNA-PEI polyplexes (31). 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 (Fig. 3). Few studies have examined the impact of N:P ratio on the size of siRNA-PEI polyplexes (24,30,32). 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-PEI (25 kDa) 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 (32). However, the opposite trend was reported for siRNA-branched-PEI (25 kDa) 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) (24). 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 (24,32), 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 (33). 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 (Fig. 4b) is particularly exciting because it maximizes the potential intracellular uptake of the polyplexes (8,30). 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 (30). Based on these promising results, future studies will evaluate these siRNA-PEI-loaded MPs in a rat TMJ disorder model (4,21) to determine their *in vivo* efficacy in alleviating TMJ inflammation.

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.

ACKNOWLEDGMENTS

We acknowledge support by the National Institutes of Health (R01 DE17441) (AGM). PMM was supported by a training fellowship from the Keck Center Nanobiology Training Program of the Gulf Coast Consortia (NIH Grant No. 5 T90 DK070121-04) for the early portions of this work and is now supported by a training fellowship from the NIH Biotechnology Training Program (NIH Grant No. 5 T32 GM008362-19). We thank Dr. Rebecca Richards-Kortum for the use of the DLS apparatus.

REFERENCES

1. Tanaka E, Detamore MS, Mercuri LG. Degenerative disorders of the temporomandibular joint: etiology, diagnosis, and treatment. J Dent Res. 2008;87:296–307.
2. Mountziaris PM, Kramer PR, Mikos AG. Emerging intra-articular drug delivery systems for the temporomandibular joint. Methods. 2009;47:134–40.
3. Fredriksson L, Alstergren P, Kopp S. Tumor necrosis factor-alpha in temporomandibular joint synovial fluid predicts treatment effects on pain by intra-articular glucocorticoid treatment. Mediators Inflamm. 2006;2006:59425.
4. Spears R, Oakes R, Moore C, Bellinger LL, Hutchins B. A determination of tumor necrosis factor expression in TMJ inflammation with the use of microarray analysis. J Dent Res. 2003;82:807–13.
5. Inoue A, Takahashi KA, Mazda O, Terauchi R, Arai Y, Kishida T, *et al.* Electro-transfer of small interfering RNA ameliorated arthritis in rats. Biochem Biophys Res Commun. 2005;336:903–8.
6. Schiffelers RM, Xu J, Storm G, Woodle MC, Scaria PV. Effects of treatment with small interfering RNA on joint inflammation in mice with collagen-induced arthritis. Arthritis Rheum. 2005;52:1314–8.
7. Kim W, Kim S. Efficient siRNA delivery with non-viral polymeric vehicles. Pharm Res. 2009;26:657–66.
8. Gary DJ, Puri N, Won Y-Y. Polymer-based siRNA delivery: Perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. J Control Release. 2007;121:64–73.
9. Mountziaris PM, Sing DC, Mikos AG, Kramer PR. Intra-articular microparticles for drug delivery to the rat TMJ. J Dent Res. 2010;89:1039–44.
10. Khan A, Benboubetra M, Sayyed PZ, Keng Wooi N, Fox S, Beck G, *et al.* Sustained polymeric delivery of gene silencing antisense ODNs, siRNA. DNAzymes and Ribozymes: *In vitro* and *in vivo* studies. J Drug Target. 2004;12:393–404.
11. Murata N, Takashima Y, Toyoshima K, Yamamoto M, Okada H. Anti-tumor effects of anti-VEGF siRNA encapsulated with PLGA microspheres in mice. J Control Release. 2008;126:246–54.
12. Jensen DMK, Cun D, Maltesen MJ, Frokjaer S, Nielsen HM, Foged C. Spray drying of siRNA-containing PLGA nanoparticles intended for inhalation. J Control Release. 2010;142:138–45.
13. Katas H, Cevher E, Alpar HO. Preparation of polyethyleneimine incorporated poly(d, l-lactide-co-glycolide) nanoparticles by spontaneous emulsion diffusion method for small interfering RNA delivery. Int J Pharm. 2009;369:144–54.
14. Woodrow KA, Cu Y, Booth CJ, Saucier-Sawyer JK, Wood MJ, Saltzman WM. Intravaginal gene silencing using biodegradable

polymer nanoparticles densely loaded with small-interfering RNA. Nat Mater. 2009;8:526–33.
15. Yuan X, Shah B, Kotadia N, Li J, Gu H, Wu Z. The development and mechanism studies of cationic chitosan-modified biodegradable PLGA nanoparticles for efficient siRNA drug delivery. Pharm Res. 2010;27:1285–95.
16. Brunner T, Cohen S, Monsonego A. Silencing of proinflammatory genes targeted to peritoneal-residing macrophages using siRNA encapsulated in biodegradable microspheres. Biomaterials. 2010;31:2627–36.
17. Cleek RL, Rege AA, Denner LA, Eskin SG, Mikos AG. Inhibition of smooth muscle cell growth *in vitro* by an antisense oligodeoxynucleotide released from poly(DL-lactic-co-glycolic acid) microparticles. J Biomed Mater Res A. 1997;35:525–30.
18. Patel ZS, Yamamoto M, Ueda H, Tabata Y, Mikos AG. Biodegradable gelatin microparticles as delivery systems for the controlled release of bone morphogenetic protein-2. Acta Biomater. 2008;4:1126–38.
19. Saraf A, Baggett LS, Raphael RM, Kasper FK, Mikos AG. Regulated non-viral gene delivery from coaxial electrospun fiber mesh scaffolds. J Control Release. 2010;143:95–103.
20. Moret I, Esteban Peris J, Guillem VM, Benet M, Revert F, Dasi F, *et al.* Stability of PEI-DNA and DOTAP-DNA complexes: effect of alkaline pH, heparin and serum. J Control Release. 2001;76:169–81.
21. Kramer PR, Puri J, Bellinger LL. Knockdown of Fcγ receptor III in an arthritic temporomandibular joint reduces the nociceptive response in rats. Arthritis Rheum. 2010;62:3109–18.
22. Fu K, Pack DW, Klibanov AM, Langer R. Visual evidence of acidic environment within degrading poly(lactic-co-glycolic acid) (PLGA) microspheres. Pharm Res. 2000;17:100–6.
23. Kerner M, Hohenberg H, Ertl S, Reckermann M, Spitzy A. Self-organization of dissolved organic matter to micelle-like microparticles in river water. Nature. 2003;422:150–4.
24. J. Nguyen, R. Reul, S. Roesler, E. Dayyoub, T. Schmehl, T. Gessler, W. Seeger, and T.H. Kissel. Amine-modified poly (vinyl alcohol)s as non-viral vectors for siRNA delivery: Effects of the degree of amine substitution on physicochemical properties and knockdown efficiency. Pharm Res. Epub ahead of print: (2010).
25. Krebs MD, Jeon O, Alsberg E. Localized and sustained delivery of silencing RNA from macroscopic biopolymer hydrogels. J Am Chem Soc. 2009;131:9204–6.
26. Bolcato-Bellemin A-L, Bonnet M-E, Creusat G, Erbacher P, Behr J-P. Sticky overhangs enhance siRNA-mediated gene silencing. Proc Natl Acad Sci U S A. 2007;104:16050–5.
27. Li Y, Breaker RR. Kinetics of RNA degradation by specific base catalysis of transesterification involving the 2'-hydroxyl group. J Am Chem Soc. 1999;121:5364–72.
28. Cao H, Jiang X, Chai C, Chew SY. RNA interference by nanofiber-based siRNA delivery system. J Control Release. 2010;144:203–12.
29. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. J Control Release. 1999;60:149–60.
30. Richards Grayson A, Doody A, Putnam D. Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery *in vitro*. Pharm Res. 2006;23:1868–76.
31. Deng R, Diao S, Yue Y, Ngai T, Wu C, Jin F. Dynamic and structural scalings of the complexation between pDNA and bPEI in semidilute and low-salt solutions. Biopolymers. 2010;93:571–7.
32. Mehrotra S, Lee I, Chan C. Multilayer mediated forward and patterned siRNA transfection using linear-PEI at extended N/P ratios. Acta Biomater. 2009;5:1474.
33. Saraf A, Hacker MC, Sitharaman B, Grande-Allen KJ, Barry MA, Mikos AG. Synthesis and Conformational Evaluation of a Novel Gene Delivery Vector for Human Mesenchymal Stem Cells. Biomacromolecules. 2008;9:818–27.