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Abstract: A low toxicity and efficient delivery system is needed to deliver small interfering RNAs (siRNA) in vitro and in vivo. The use of mesoporous silica nanoparticles (MSN) is becoming increasingly common due to its biocompatibility, tunable pore size and customizable properties. However, bolus delivery of siRNA/MSN complexes remains suboptimal, especially when a sustained and long-term administration is required. Here, we utilized electrospun scaffolds for sustained delivery of siRNA/MSN-PEI through surface adsorption and nanofiber encapsulation. As a proof-of-concept, we targeted collagen type I expression to modulate fibrous capsule formation. Surface adsorption of siRNA/MSN-PEI provided sustained availability of siRNA for at least 30 days in vitro. As compared to conventional bolus delivery, such scaffold-mediated transfection provided more effective gene silencing ($p < 0.05$). On the contrary, a longer sustained release was attained (at least 5 months) when siRNA/MSN-PEI complexes were encapsulated within the electrospun fibers. In vivo subcutaneous implantation and biodistribution analysis of these scaffolds revealed that siRNA remained localized up to $\sim 290 \mu\text{m}$ from the implants. Finally, a fibrous capsule reduction of $\sim 45.8\%$ was observed after 4 weeks in vivo as compared to negative scrambled siRNA treatment. Taken together, these results demonstrate the efficacy of scaffold-mediated sustained delivery of siRNA/MSN-PEI for long-term non-viral gene silencing applications.

**Sustained delivery of siRNA/mesoporous silica nanoparticle (siRNA/MSN)
complexes from nanofiber scaffolds for long-term gene silencing**

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Keywords

Electrospinning, gene silencing, host-implant integration, foreign body reaction

Abstract

A low toxicity and efficient delivery system is needed to deliver small interfering RNAs (siRNA) *in vitro* and *in vivo*. The use of mesoporous silica nanoparticles (MSN) is becoming increasingly common due to its biocompatibility, tunable pore size and customizable properties. However, bolus delivery of siRNA/MSN complexes remains suboptimal, especially when a sustained and long-term administration is required. Here, we utilized electrospun scaffolds for sustained delivery of siRNA/MSN-PEI through surface adsorption and nanofiber encapsulation. As a proof-of-concept, we targeted collagen type I expression to modulate fibrous capsule formation. Surface adsorption of siRNA/MSN-PEI provided sustained availability of siRNA for at least 30 days *in vitro*. As compared to conventional bolus delivery, such scaffold-mediated transfection provided more effective gene silencing ($p < 0.05$). On the contrary, a longer sustained release was attained (at least 5 months) when siRNA/MSN-PEI complexes were encapsulated within the electrospun fibers. *In vivo* subcutaneous implantation and biodistribution analysis of these scaffolds revealed that siRNA remained localized up to ~290 μm from the implants. Finally, a fibrous capsule reduction of ~45.8 % was observed after 4 weeks *in vivo* as compared to negative scrambled siRNA treatment. Taken together, these results demonstrate the efficacy of scaffold-mediated sustained delivery of siRNA/MSN-PEI for long-term non-viral gene silencing applications.

1. Introduction

By inhibiting the expression of targeted genes [1–3], small interfering RNAs (siRNA) are considered as promising therapeutics for the treatment of pathologies such as cancer [4,5] and genetic disorders. Although less widely used, siRNAs also display potential in tissue engineering and regenerative medicine [6]. These molecules showed promising effects in the regeneration of tissues such as bones [7], muscles and connective tissues [8], for wound healing [9] and in directing cell fate.[10] However, the use of siRNA as a therapeutic agent has been hindered by its poor cellular uptake and short half-life.[11] To overcome siRNA degradation by RNases and improve intracellular delivery, extensive efforts have been made to develop new carriers.[10,12] In particular, mesoporous silica nanoparticles (MSNs) have received increasing attention.[13]

Besides being biocompatible,[14–16] MSN possesses pore sizes and structures that can be tuned to achieve higher drug loading efficiencies. The use of MSN as a carrier for siRNA is particularly interesting since the presence of chemical groups on the surface of these nanoparticles permit the introduction of functionalities to provide control over target-selective delivery, cellular uptake, drug release rates and the extent of endosomal escape.[5,17–19]

Thus far, most studies have described the systemic and bolus delivery of siRNA/MSN complexes to silence protein agonists of tumoral processes as cancer treatments.[18,20–22] However, such siRNA systemic or bolus delivery is generally short-lived. In applications that require sustained availability of siRNA, e.g. tissue

1 regeneration, these approaches would require multiple injections/doses, which may lead
2 to systemic side effects.

3
4 A promising approach to attain sustained delivery of siRNA/MSN complexes is to
5 incorporate them into scaffolds. As compared to bolus delivery, gene delivery from a
6 scaffold offers more controllable and localized transfection.[3] This method not only
7 minimizes systemic side effects but also enhances gene transfection efficiency [23,24]
8 and provides long term gene silencing [25]. However, to date, scaffold-mediated
9 delivery of siRNA/MSN complexes has not been reported.

10
11 In this study, we developed novel fiber constructs for controlled delivery of
12 siRNA/MSN-PEI complexes. Specifically, we explored the feasibility of fiber scaffold-
13 mediated delivery of siRNA/MSN-PEI complexes for sustained non-viral gene silencing.
14 Besides mimicking the architecture of the natural extracellular matrix, fiber scaffolds
15 also promote tissue integration.[26] Correspondingly, these constructs are excellent
16 frameworks for tissue engineering applications. Here, we evaluated two siRNA/MSN-
17 PEI complex incorporation methods: 1) Poly (ϵ -caprolactone) (PCL) fiber surface
18 absorption using mussel-inspired bioadhesive and 2) direct encapsulation within poly
19 (caprolactone-co-ethyl ethylene phosphate) (PCLEEP) nanofibers. While both methods
20 provided sustained release of drugs,[23,27–31] major differences exist. In particular, the
21 surface adsorption method typically results in a larger initial burst release of drugs,
22 making it an effective method for *in vitro* transfection.[30,32,33] On the other hand,
23 nanofiber encapsulation provides more sustained/prolonged drug release. Importantly,

by encapsulating siRNA within the polymer matrix, these labile molecules were protected from enzymatic degradation and are, therefore, ideal for *in vivo* applications.[25]

Here, we developed biodegradable MSN-PEI to allow cellular uptake of siRNA/MSN-PEI complexes. Then, we compared the gene-silencing efficacy between conventional bolus delivery vs. the two scaffold-mediated methods of delivery. We showed that nanofiber scaffold-mediated non-viral delivery of siRNA/MSN-PEI successfully down-regulated the expression of collagen 1 *in vitro* and *in vivo*. Such scaffolds may find useful applications for long-term non-viral gene silencing in regenerative medicine.

2. Material and method

2.1 Material

Poly (ϵ -caprolactone) (PCL, M_w : 70 kDa), 3,4-dihydroxy-L-phenylalanine (DOPA), 2,2,2-trifluoroethanol (TFE), chloroform, 100 % ethanol, heparin, 10 % formalin, triton-X 100, fluoromount, triethanolamine (TEA), (3-glycidyloxypropyl)trimethoxysilane (3-GPS), branched polyethylenimine (PEI, M_w = 800), and cetyltrimethylammonium p-toluenesulfonate (CTAT) were purchased from Sigma-Aldrich. Donkey anti-goat IgG Alexa Fluor 488 (A-11055), Silencer[®] Select Negative Control siRNA (4390843-siNEG), Silencer[®] Select PPIB (S10908-siCyB), Silencer[®] Select COLA1 siRNA targeting the human (S3276-siCOLL1A 1) and the rat (S131320-siCOLL1A 1), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), Live/Dead[®] kit, Penicillin–

streptomycin (10,000 U ml/ml), antibiotic–antimycotic and PBS (pH 7.4) were purchased from ThermoFisher. The transfection reagent, TransIT-TKO®, was obtained from Mirusbio, USA. Ribogreen[®] reagent, Trizol reagent, 4',6-Diamidino-2-Phenylindole (DAPI), Alexa Fluor 488 Phalloidin (AF488 Phalloidin) were purchased from Invitrogen. Tris-EDTA (TE) buffer (pH 8.0) and 10X PBS were purchased from First Base, Singapore. Cy5-labeled double-stranded oligonucleotides (Cy5-RNA) was purchased from Integrated DNA Technologies. Goat anti-human collagen type I (L19- sc-8783) was purchased from Santa Cruz. Human dermal fibroblasts (HDFs) were purchased from Lonza, Basel, Switzerland. dNTP Mix and M-MLV reverse transcriptase oligo(dT) primer were purchased from Promega. Tetraethoxysilane (TEOS), ethanol, bis[3-(triethoxysilyl)propyl]tetrasulfide (BTES), and 1,4-dithio-DL-threitol (DTT, 98 %) were obtained from Alfa Aesar. Hydrochloric acid (HCl, 37 %) was from Acros Organics. Paraformaldehyde was obtained from Millipore and stainless steel surgical wound clips from Fine Science Tools, Canada. Sprague-Dawley rats were obtained from In Vivos Pte Ltd (Singapore).

2.2 Synthesis of PEI modified mesoporous silica nanoparticles (MSN-PEI)

The biodegradable MSNs were synthesized according to a previously reported method with modifications.[34] Typically, CTAT (1.0 g) and TEA (0.4 mL) were dissolved in 50.0 ml water and stirred at 80 °C for 2 h. Then, a mixture of BTES (0.6 ml) and TEOS (0.8 ml) was added dropwise, and the reaction was performed at 80 °C for another 4 h. Subsequently, the solution was cooled to room temperature while stirring. The product was collected by centrifugation, washed with ethanol and water three times

1 and dried under vacuum. Then, 0.18 g of as-prepared sample was extracted thrice with
2 a solution of 1 ml of HCl (37 %) and 25 ml of ethanol by refluxing at 80 °C for 12 h to
3 efficiently remove the CTAT template. Finally, the nanoparticles were washed with
4 water three times, and dried under vacuum.

5
6 The epoxysilane functionalization of MSN was achieved according to a reported
7 procedure with minor modifications.[35] Briefly, the obtained MSNs (130 mg) were
8 dispersed in toluene and sonicated for 90 min. After that, 3-GPS (0.2 ml) was added,
9 and the mixture was stirred for 2 h at room temperature. Then, the reaction solution was
10 refluxed at 80 °C for 24 h. The product was separated by centrifugation and washed
11 with ethanol and water three times. The final product was obtained as a white powder
12 after lyophilization.

13
14 Epoxysilane functionalized MSN powder (58 mg) was then suspended in pure
15 water (30 mL) by sonication (30 min). PEI ($M_w = 800$) solution (0.3 ml, 100 mg/ml) was
16 added, and the reaction was performed at room temperature for 24 h. After
17 centrifugation and wash with water, PEI modified MSNs were redispersed in water for
18 further studies.

19 20 **2.3 Degradability of mesoporous silica nanoparticles**

21 100 µg of MSN-PEI was dispersed in 1.5 ml phosphate buffer (50 mM, pH 7.4)
22 with 20 mM DTT and incubated at 37 °C with constant shaking (200 rpm). After 72 h,
23 the suspension was centrifuged at 15,000 rpm for 10 min, washed with water three

times (to remove the salt), and the obtained product was used for transmission electron microscopy (TEM, JEOL 2100; accelerating voltage: 200 kV) characterization.

2.4 Scaffold fabrication

2.4.1 siRNA/MSN-PEI complexation

siRNA was reconstituted in RNase-free water to obtain a stock concentration of 100 μ M. Meanwhile, the solution of MSN-PEI (6 mg/ml) was sonicated for 15 min before use. Thereafter, siCOLL1A 1 or siCyB were complexed with MSN-PEI at two different ratios (1:20 and 1:40, w/w) in 100 μ l of fresh DMEM. 2 μ g and 4 μ g were prepared to be loaded onto coated-scaffolds of 3.7 ± 0.16 mg or 11 μ g were prepared to be mixed with 500 μ l of PCLEEP to fabricate 6 encapsulated-scaffolds. As a control, similar amount of siNEG (scrambled siRNA) and siColl1A 1 were complexed with either MSN-PEI or TKO[®] at 1:1 (v/v) ratio. After 25 min of complexation at room temperature the complexes were incorporated with the scaffolds.

2.4.2 Fabrication of siRNA/MSN-PEI-coated scaffolds

PCL was dissolved at 15 % (w/v) in TFE. The homogenous solution was then loaded into a syringe with a 21 G needle and fixed on a syringe pump (KDS-100, KS scientific). For electrospinning, a voltage of +12 kV was applied to the polymer solution, which was dispensed at a flow rate of 1.5 ml/h. The fibers were collected on a negatively charged (-4 kV) rotating target (1200 rpm) that was placed 15 cm from the syringe needle.

PCL fiber scaffolds were cut to fit 24 well plates (1.9 cm²) and pre-wet quickly in a solution of ethanol/water (70/30 v/v). Thereafter, they were rinsed and incubated in deionized water (DI water) overnight. The following day, the scaffolds were incubated in 0.5 ml of DOPA solution at 0.5 mg/ml in a pH 8 buffer that comprised of bicine (10 mM) and NaCl (50 mM). After 5 h of coating under 120 rpm agitation, the samples were rinsed briefly and incubated for 1 h in DI water under 120 rpm agitation and then lyophilized overnight. All samples were sterilized under UV for 45 min. Lastly, 2 or 4 µg of siRNA/MSN complexes were loaded onto the fibers for surface absorption at 37 °C for 30 min.

2.4.3 Fabrication of siRNA/MSN-PEI-encapsulated scaffolds

To increase scaffold hydrophilicity and therefore, the release rate, poly (caprolactone-co-ethyl ethylene phosphate) copolymers (50:1) (PCLEEP, *M_w*: 70 kDa), were utilized for the encapsulation of siRNA complexes. PCLEEP was synthesized through ring opening polymerization of ε-caprolactone and ethyl ethylene phosphate as reported previously.[36,37] From there, PCLEEP was dissolved in TFE to obtain a 36 % (w/v) homogenous solution. Thereafter 11 µg of siCOLL1A1 were complexed to MSN-PEI (1:40) or TKO[®] (1:1) as described above, and 11.2 µl of EDTA was added to the complex followed by RNase -free water to constitute the final volume of the complex solution to 100 µl. The complex solution was then mixed thoroughly with 500 µl of PCLEEP for electrospinning. Thereafter, the homogenous solution of siCOLL1A1/MSN-PEI and PCLEEP was electrospun following the same parameters as highlighted in section 2.4.2.

2.5 Evaluation of scaffold morphology and drug distribution

All scaffolds were sputter-coated with platinum (JEOL, JFC-1600) at 10 mA for 120 s and observed under the scanning electron microscope (SEM; JEOL, JSM-6390 LA, Japan) at an accelerating voltage of 10 kV.

To evaluate the distribution of siRNA on or inside the fibers, Cy5-labeled RNA (similar size as siRNA) was used. After complexation with MSN-PEI, Cy5-RNA/MSN-PEI complexes were incorporated as described in section 2.4.1.2. and 2.4.2. The distribution of Cy5-RNA/MSN-PEI complexes was analyzed using a fluorescence microscope (Leica DMI8) after the samples were washed 3 times for 5 min in PBS.

2.6 Quantification of siRNA loading and release

Each scaffold (3.7 ± 0.16 mg) was incubated in 1.2 ml of 1X TE buffer at 37 °C. At each time point, 600 µl of buffer was collected and replaced by an equal volume of fresh buffer. In addition, the decomplexation of siRNA from MSN-PEI or TKO® was achieved by mixing 100 µl of 100 mg/ml heparin with the supernatant. After shaking at 130 rpm for 1 h, this solution of decomplexed siRNA was used to measure the amount of siRNA that was released using the Quant-iT™ RiboGreen RNA reagent kit, following manufacturer's protocol. The percentage cumulative release of siRNA was then presented as mean $\pm$ standard deviation (S.D.).

The experimental loading efficiency of siRNA was evaluated based on previous protocols.[23,24] Briefly, siRNA/MSN-PEI- and siRNA/TKO-encapsulated nanofiber scaffolds (n = 3) were cut into halves and dissolved in 1 ml of chloroform. Thereafter, 200 µl of TE buffer was added. After shaking for 30 min, the aqueous phase was collected. This process was repeated three times. The amount of siRNA within the extracted aqueous solution was finally determined using RiboGreen assay. The efficiency of extracting siRNA from PCLEEP solution was also accounted for by doping PCLEEP solutions with known amounts of siRNA/MSN-PEI or siRNA/TKO mixtures. Thereafter, the polymer/siRNA/MSN-PEI or polymer/siRNA/TKO mixtures were placed under vacuum overnight at room temperature to remove all solvents. Next, dry polymer/siRNA/MSN-PEI or polymer/siRNA/TKO constructs (n = 3) were cut into halves and dissolved in 1 ml of chloroform each. siRNA was finally extracted and quantified as described above to obtain extraction efficiencies. The experimental loading efficiencies were finally computed using the following equation:

$$\text{Loading efficiency} = \frac{\text{Total siRNA released} + \text{Total siRNA extracted}}{\text{Total theoretical siRNA loading}} \times 100\%$$

The state of complexation of siRNA after 33 and 150 days of release was verified by measuring the amount of siRNA within the supernatant using the QuantiTTMRiboGreen RNA reagent kit, with and without heparin decomplexation as described above.

2.7 Cellular uptake study

HDFs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) that was supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin. Cells were maintained at 37 °C with 5 % CO₂. The extent of cellular uptake of the siRNA/MSN-PEI complexes was evaluated by using Cy5-RNA (similar size as siRNA) in place of siRNA for visualization.

2.7.1 Conventional bolus delivery

For bolus transfection, cells were seeded at a density of 8×10^3 cells/cm² in 24-well plates one day before transfection in 300 µl of medium. The next day, siRNA was complexed with MSN-PEI at two different ratios: 1:20 and 1:40 (w/w) and TKO® (1:1, v/v) to reach a final concentration of 50 nM and 100 nM in the culture medium. The complex was then added into the well and mixed thoroughly with the medium. The medium was replaced with fresh medium after 24 h, and cellular uptake was evaluated 48 h after transfection. Specifically, cells were fixed with 4 % paraformaldehyde for 15 min and permeabilized in 0.3 % Triton-X 100 for 10 min. Thereafter, all samples were stained with AF488 Phalloidin (1:500) for 1 h at room temperature and DAPI (1:1000) for 5 min. The extent of cellular uptake of Cy5-RNA was then evaluated using an inverted fluorescence microscope (Leica DMI8). To quantify the intensity of Cy5-RNA within the cells, all images were taken using the **exact** same parameters. The intensity of the signals inside each cell was then quantified using the Image J software. The measurement was repeated for three samples per group.

2.7.2 siRNA/MSN-PEI-coated scaffolds

Cells were seeded at a density of 8×10^3 cells/cm² onto the Cy5-RNA/MSN-PEI-incorporated fiber scaffolds in 24-well plates with 300 μ l of medium. Teflon rings were used to hold the scaffolds in position at the bottom of each well. Thereafter, cellular uptake was evaluated 72 h after cells seeding, following the same protocol as described in section 2.7.1.

2.7.3 siRNA/MSN-PEI-encapsulated scaffolds

Cellular uptake study on Cy5-RNA/MSN-PEI-encapsulated scaffolds was evaluated at 3, 7 and 14 days after seeding cells onto siRNA/MSN-PEI (1:40)-encapsulated scaffolds following the same protocol as described in section 2.7.2.

The viability of the cells was tested after 3, 7 and 14 days on siRNA/MSN-PEI (1:40)-encapsulated scaffolds using Live/Dead® assay following the manufacturer's protocol.

2.8 *In vitro* gene silencing evaluation

The *in vitro* gene silencing efficacy was evaluated after bolus delivery as well as after delivery that was mediated by coated scaffolds and encapsulated scaffolds following the same protocol. Briefly, HDFs were seeded at a density of 2.5×10^4 cells/cm² in 24 well plates and onto each scaffold. After 72 h, HDFs were lysed with Trizol® reagent and RNA was extracted following manufacturer's protocol. Then, cDNA was obtained using Oligo(dT) primers and M-MLV Reverse Transcriptase. Real-time PCR was performed using the StepOnePlus real-time PCR system (Applied Bio-

systems) using the comparative Ct method after ensuring that all primers had similar amplification efficiencies. The primer sequences for collagen I (COL1A1) was forward 5'-CAATGCTGCCCTTTCTGCTCCTTT-3' and reverse 5'-ATTGCCTTTGATTGCTGGGCAGAC-3'. After checking the stability of β -actin expression across all sample groups, it was chosen to be the housekeeping gene. The primer sequences were: forward 5'-GGCACCCAGCACAATGAAGATCAA-3' and reverse 5'-ACTCGTCATACTCCTGCTTGCTGA-3'. The real-time PCR conditions used were as follows: 10 min at 95 °C, 40 cycles at 95 °C for 15 s, followed by 60 °C for 1 min. The results of mRNA expression were then normalized to the respective control in which HDFs were cultured on plate or scaffolds and transfected with siNEG/MSN-PEI complexes. Three independent repeats were conducted for each experiment.

2.9 Immunostaining

The collagen produced by the transfected cells was immunostained after 3, 7 and 14 days of culture. Briefly, samples were fixed with 10 % formalin solution for 1 h at room temperature. Non-specific sites were then blocked in a blocking solution (1 % BSA in PBS) for 3 h. Cells were then incubated overnight with goat anti-collagen type I antibody (1:50) in the blocking solution at 4° C. After washing three times with PBS, collagen was revealed by incubation for 2 h with Alexa Fluor 488 donkey anti-goat antibody (1:200) in PBS. Nuclei were counterstained with DAPI (1:1000). The samples were then mounted on glass slides using Fluoromount G and imaged with a confocal microscope (Zeiss LSM 800). Untreated scaffolds (plain scaffolds) were used as negative control.

2.10 In vivo studies

Cy5-RNA/MSN-PEI- and siCOLL1A/MSN-encapsulated scaffolds were used to evaluate the *in vivo* biodistribution of MSN-PEI complexes and *in vivo* silencing efficiency respectively. The average thickness of the PCLEEP scaffolds was 200.2 ± 5.4 μm . All materials were sterilized using UV irradiation for 1 h prior to implantation.

2.10.1 Surgical procedure

The animal care and experimental procedures were carried out in accordance with the Institutional Animal Care and Use Committee (IACUC, NTU) guidelines. Adult female Sprague-Dawley rats (8 weeks old) were housed under temperature controlled conditions, with a normal 12:12 h light: dark cycle with ad libitum access to food and water.

Table 1 summarizes all the siRNA/MSN-PEI-encapsulated scaffolds that were used for the *in vivo* studies, along with their notation, processing parameters, time point and number of animals per group. 9 animals were used to study the siRNA/MSN-PEI biodistribution, and 8 animals were used to evaluate the silencing efficacy of the scaffolds.

Animal studies were conducted according to our previous protocol. [25] Briefly, rats were anesthetized by inhalation of 4-5 % mixture of Aerrane[®] isoflurane with air. Then, a mixture of Ketamine (73 mg/kg) and Xylazine (10 mg/kg) was injected

1 intraperitoneally. Following that, the backs were shaved and cleaned with 70 % ethanol
2 and betadine. Two parallel skin incisions (1.2 cm) were then made, each located beside
3 the spinal column and 2 cm above the tail. Another two skin incisions were made 4 cm
4 apart from the posterior incisions. Thereafter, subcutaneous pockets were created using
5 surgical scissors. Scaffolds (1 × 2 cm) were then introduced through the incision into the
6 pocket and away from the incision site. The skin was then closed with 11 mm stainless
7 steel surgical wound clips. Following that, Neosporin ointment was applied onto the
8 incision to minimize potential post-operative discomfort. To randomize the samples
9 distribution, each animal received all 4 groups of scaffold implants, which were placed in
10 no particular order.

11 12 **2.10.2 Biodistribution evaluation**

13 At 3 days, 1 and 2 weeks post-operation, animals were perfused with 0.9 %
14 saline followed by 4 % ice-cold paraformaldehyde. After perfusion, the tissue enclosing
15 the implants (2 × 3 cm), kidney and liver were carefully removed, post fixed for 2 h at
16 4°C and then sequentially transferred to 15 % sucrose and 30 % sucrose after 24 and
17 48 h, respectively. All samples were stored at 4 °C until sectioned. Samples were then
18 sectioned on a cryostat at 20 µm thickness. Four sections per condition were used to
19 evaluate the Cy5-RNA distribution using an inverted fluorescence microscope (Leica
20 DMI8) and a confocal microscope (Zeiss LSM 800).

2.10.3 *In vivo* evaluation of fibrous capsule formation

At 2 and 4 weeks post-implantation, the implanted scaffold, along with some of its surrounding tissue (2 × 3 cm) was carefully removed and fixed in 10 % neutral buffered formalin (NBF) for 24 h. Thereafter, samples were placed in an automatic vacuum tissue processor (Leica ASP6025) before being embedded into paraffin blocks. From these blocks, sections of 4 µm thickness were obtained, mounted onto Superfrost Plus glass slides and stained with both Hematoxylin and Eosin (H&E) and Masson's Trichrome. Stitched images for quantifying fibrous capsule thickness were acquired using an inverted microscope (Leica DMI8). Measurements were taken at 13 random regions along the periphery of the scaffold on Masson's Trichrome stained sections. Four sections were analyzed for each group, giving a total of 52 measurements for each time point.

2.11 Statistical analysis

All results correspond to separate experiments done in triplicates and values are given as mean ± standard deviation. Statistical analyses were performed with Prism5 software. Non-parametric Wilcoxon test was used to compare the different groups.

3. Results

3.1 Degradation of MSN-PEI

Bioreductive MSN-PEI was obtained by introducing BTES in the composition of the particles, as illustrated in Figure 1A. After PEI modification, the average diameter of MSN-PEI particles was 132 ± 1 nm (Figure 1B). The degradability of the nanoparticles

was verified by incubating MSN-PEI in 20 mM DTT, a reducing agent for breaking disulfide linkages. As shown in Figure 1C, after 72 h, the substantial structure of MSN-PEI collapsed and broke down into smaller pieces, demonstrating the biodegradability of the nanocarriers in a reductive condition that mimics the intracellular microenvironment.

3.2 Characterization of siRNA/MSN complexes

As shown in Supplementary Figure 1, among the different ratios of siRNA/MSN-PEI (w/w) tested, the minimal ratio that was required to fully complex all siRNA was 1:20. Therefore, a ratio of 1:20 and 1:40 were used for subsequent studies.

As reported in Table 2 and indicated by zetasizer measurements, the initial sizes of MSN-PEI increased after complexation with siRNA, reaching values of 705 ± 24 nm and 588 ± 16 nm for 1:20 and 1:40 ratios respectively. Additionally, the zeta potential of uncomplexed MSN-PEI, 1:20 and 1:40 complexation ratios were 54.0 ± 0.9 mV, 16.3 ± 2.9 mV and 20.6 ± 2.2 mV respectively.

3.3 Morphology of siRNA/MSN-PEI-coated and -encapsulated scaffolds

3.3.1 siRNA/MSN-PEI-coated scaffolds

As indicated in Figure 2A, a uniform distribution of nucleic acid, as indicated by Cy5-RNA, was obtained on the surfaces of the PCL fibers. Figure 2B shows the representative SEM image of siRNA/MSN-PEI-coated scaffolds. While plain PCL fibers had an initial average diameter of 1.03 ± 0.29 μ m, (1:40) siRNA/MSN-PEI-coated fibers had a higher average diameter (1.36 ± 0.12 μ m) after the incorporation of the

1 complexes. After 30 days of incubation in PBS, the coating of complexes was no longer
2 detectable on the fibers and the fiber morphology was similar to that before drug loading
3 (Figure 2C and Supplementary Figure 2).

5 3.3.2 siRNA/MSN-PEI-encapsulated scaffolds

6 As demonstrated in Figure 2D, Cy5-RNA signals remained detectable along the
7 polymer fibers even after washing 3 times with PBS. This indicated that the
8 siRNA/MSN-PEI complexes were successfully encapsulated within the fibers. As shown
9 in Figure 2E, after encapsulation of siRNA, PCLEEP fibers presented an average
10 diameter of 785 ± 132 nm which was similar to that of the plain fibers (798 ± 198 nm)
11 (Supplementary Figure 2). After 150 days of incubation in PBS at 37 °C, the morphology
12 of the scaffolds remained unaffected by the diffusion of siRNA from the fibers (Figure 2F
13 and Supplementary Figure 2).

15 3.4 SiRNA release kinetics studies

16 3.4.1 siRNA/MSN-PEI-coated scaffolds

17 As shown in Figure 2G, siRNA in all samples had a burst release (~ 40 % within
18 the first hour). SiRNA/MSN-PEI (1:20) complexes were released at the fastest rate,
19 followed by 1:40 complexes and finally siRNA/TKO[®] complexes (73 % vs 51 % vs 39 %
20 within 2 days respectively). In fact, after the initial burst release, the delivery of
21 siRNA/TKO[®] complex was delayed slightly for 2 days. Finally, after 30 days, 90 %
22 (siRNA/TKO[®]) to 100 % (MSN-PEI 1:20 and 1:40) of siRNA were released from all
23 scaffolds. The loading efficiencies for siRNA/TKO[®], siRNA/MSN-PEI (1:20) and

1 siRNA/MSN-PEI (1:40) scaffolds were 90.8 ± 8.2 , 96.5 ± 3.6 and 97.3 ± 5.0 %,
2 respectively, suggesting that TKO[®] and MSN-PEI complexation did not affect drug
3 loading efficiency.

4 5 **3.4.2 siRNA/MSN-PEI-encapsulated scaffolds**

6 A sustained release of siRNA was observed from siRNA/MSN-PEI (1:40)-
7 encapsulated scaffolds (Figure 2H) for at least 5 months *in vitro*. Initially, siRNA was
8 released with a slight burst (less than 10 %). Thereafter, a release of 55.58 % was
9 observed over the course of 5 months. On the other hand, the rate of siRNA release
10 from siRNA/TKO[®] encapsulated scaffolds was quicker. In particular, 67 % of siRNA was
11 released within 17 days before reaching a plateau phase after 48 days. As shown in
12 Figure 2I, decomplexation with heparin increased the amount of siRNA that was
13 detected by the RiboGreen assay. This indicated that the siRNA within the supernatant
14 remained at least partially as complexes with MSN-PEI after 33 and 150 days of
15 release. The loading efficiencies of scaffolds that encapsulated siRNA/MSN-PEI and
16 siRNA/TKO[®] complexes were 85.47 ± 11.89 and 80.79 ± 12.1 % respectively. This
17 suggested that TKO[®] and MSN-PEI complexation did not greatly affect the drug loading
18 efficiencies.

19 20 **3.5 Cellular uptake study**

21 **3.5.1 Conventional bolus delivery**

22 As shown in Supplementary Figure 3, 92.3 to 95.4 % of HDFs took up
23 siRNA/MSN-PEI complexes. Thereafter, the siRNA continued to associate with MSN

after being endocytosed (Supplementary Figure 4). The impact of siRNA/MSN-PEI ratio on cellular uptake efficiency in HDFs after 3 days is illustrated in Figure 3A and quantified as shown in Figure 3D. An increase in fluorescence signals in cells was observed with an increase in the concentration of Cy5-RNA and MSN-PEI ratio. Correspondingly, the highest extent of cellular uptake was obtained with 100 nM of RNA/MSN-PEI (1:40) samples. As compared to cells that were transfected with TKO[®], the fluorescent intensity of Cy5-RNA was higher inside the cells when the RNA was delivered by MSN-PEI. Moreover, rounded cells were observed in the presence of TKO[®], suggesting cytotoxicity of TKO[®] (Supplementary Figure 3).

3.5.2 siRNA/MSN-PEI-coated scaffolds

Figure 3B shows the cellular uptake efficiency of siRNA/MSN-PEI by HDFs on siRNA/MSN-PEI-coated scaffolds. Similar to bolus delivery, an increase in fluorescence was observed with an increase in the quantity of Cy5-RNA and MSN-PEI ratio (Figure 3D). Therefore, the highest uptake was obtained with 4 µg of RNA/ MSN-PEI (1:40) samples. As compared to siRNA delivery using MSN-PEI, weaker Cy5-RNA signals were detected in cells when TKO[®] was used as the delivery vector. In addition, significantly higher Cy5- RNA signals were detected in cells that were transfected using siRNA/MSN-PEI-coated scaffolds as compared to bolus delivery ($p < 0.05$) (Figure 3D).

3.5.3 siRNA/MSN-PEI-encapsulated scaffolds

As shown in Figure 3C, HDFs took up siRNA/MSN-PEI complexes after 3, 7 and 14 days of culture on siRNA/MSN-PEI-encapsulated scaffolds. In particular, the

fluorescent signals increased significantly with cell culture time. However, the fluorescence intensity of Cy5-RNA within the cells was significantly lower ($p < 0.05$) than siRNA/MSN-PEI-coated scaffolds and bolus delivery (Figure 3D). Cell viability percentage after 3, 7 and 14 days on siRNA/MSN-PEI-encapsulated scaffolds was verified and found to be between 80 to 98 %, similar to untreated scaffolds and siNEG/MSN-PEI-encapsulated scaffolds, suggesting that MSN-PEI is not cytotoxic (Supplementary Figure 5).

3.6 In vitro gene silencing evaluation

3.6.1 Collagen silencing with conventional bolus delivery of siRNA/MSN-PEI

As shown in Figure 4, bolus delivery of siCOLL1A1/MSN-PEI (1:40) significantly decreased collagen 1A1 expression as compare to siNEG/MSN-PEI (1:40). The silencing efficiency increased with siCOLL1A1 concentration. Specifically, 68.9 and 85.5 % of silencing efficiency (as compared to negative control) were observed after bolus delivery of 50 nM and 100 nM of siCOLL1A1, respectively. On the other hand, collagen expression of HDFs seeded on siCOLL1A1/MSN-PEI (1:20) samples was not significantly different from siNEG/MSN-PEI (1:20) control. These results demonstrated that the MSN-PEI ratio is an important parameter to ensure efficient gene silencing of collagen 1A1. Although siNEG/TKO did not silence collagen expression, as expected, it appeared that siNEG/MSN (1:20) and (1:40) down regulated collagen expression, suggesting an intrinsic collagen 1A1 silencing capability of the MSN-PEI.

MSN-PEI seemed to influence the silencing of collagen expression. Specifically, collagen expressions in HDFs transfected with siNEG/MSN-PEI (1:20), and siNEG/MSN-PEI (1:40) were silenced with an efficiency of 49.1 % and 21.9 % respectively as compared to collagen expression by HDFs without treatment (untreated cells). This effect is not due to siNEG non-specific reaction since the siNEG/TKO complex did not appear to alter collagen expression as compared to untreated cells.

3.6.2 siRNA/MSN-PEI-coated scaffolds

As shown in Figure 5A, similar to bolus delivery, HDFs collagen silencing increased with 1:40 complexation ratio. Specifically, HDFs seeded directly on 4ug-siCOLL1A1/MSN-PEI (1:40) scaffolds showed significant gene silencing (93.0 % of silencing efficiency) as compared to 4ug-siCOLL1A1/MSN-PEI (1:20) (72.6 % of silencing efficiency).

In addition, collagen silencing efficiency also increased with siCOLL1A quantity for fixed MSN-PEI ratios. In particular, the extent of collagen silencing was higher when cells were seeded on 4ug-siCOLL1A1/MSN-PEI (1:40) than on 2ug-siCOLL1A1/MSN-PEI (1:40) with 93.0 and 76.3 % of silencing efficiency respectively. In contrast, the silencing efficiency of 2ug-siCOLL1A1/TKO[®] samples was only of 19.7 %. As already described for bolus delivery, MSN-PEI seemed to influence collagen silencing. Collagen silencing was observed in cells seeded on 2ug-siNEG/MSN-PEI (1:40) as compared to the untreated scaffold (Plain scaffolds). These trends were further confirmed at the protein level by immunostaining for the secreted collagen 1A by HDFs as shown in

Figure 5B. To further demonstrate the silencing capability of siRNA/MSN-PEI complexes, we also targeted Cyclophilin B (CyB) as a model high abundance protein. The silencing of CyB gene with sicyB/MSN-PEI-coated scaffolds showed similar trends as collagen silencing as shown in Supplementary Figure 6.

3.6.3 siRNA/MSN-PEI-encapsulated scaffolds

HDFs seeded on siCOLL1A/MSN-PEI-encapsulated scaffolds showed effective collagen silencing as compared to siNEG/MSN-PEI-encapsulated scaffolds (Figure 6A). Although no Coll1A1 mRNA fold change was observed after 3 days on siCOLL1A/MSN-PEI-encapsulated scaffolds, silencing efficiencies of 40.6 % and 34.6 % were observed after 7 and 14 days, respectively. The effective collagen silencing that was mediated by siCOLL1A/MSN-PEI-encapsulated scaffolds after 7 and 14 days were also observed at the protein level, as shown in Figure 6B. In comparison, similar silencing efficiency (38.9 %) was observed only at a later time point of Day 14 when HDFs were seeded on siCOLL1A/TKO[®]-encapsulated scaffolds. The extent of gene silencing of CyB was also evaluated for siRNA/MSN-PEI-encapsulated scaffolds. As shown in Supplementary Figure 6, similar trends were observed when HDFs were seeded on siCyB/MSN-PEI-encapsulated scaffolds.

3.7 In vivo studies

Due to the sustained siRNA/MSN-PEI delivery that was provided by encapsulated scaffolds, siCOLL1A1/MSN-PEI- and siCOLL1A1/TKO[®]-encapsulated scaffolds were subsequently assessed for their *in vivo* efficacy.

3.7.1 Biodistribution evaluation

The *in vivo* biodistribution of siRNA/MSN-PEI was evaluated using Cy5-RNA/MSN-PEI-encapsulated scaffolds after 3, 7 and 14 days of subcutaneous implantation in rats, since both Cy5-RNA and siRNA are similar in structure (21-23 bp RNA molecules). Figure 7 shows the scaffold-tissue interface at each time point. After 3 days, no fluorescence was detected outside the scaffolds. However, after 7 days, Cy5-RNA signals were observed in the surrounding tissues (fluorescence marked with white arrow). The signals became more diffused after 14 days, with Cy5-RNA signals being detected up to $291.7 \pm 41.3 \mu\text{m}$ from the implant. In contrast, no fluorescence was observed in the livers and kidneys of all animals suggesting that the siRNA was only released locally into the surrounding tissues.

3.7.2 In vivo evaluation of fibrous capsule formation

Figure 8 shows the Masson's Trichrome and H&E histological evaluations. As indicated by the Masson's Trichrome staining, a capsule was formed around the scaffolds after 2 and 4 weeks of implantation. A detailed quantification revealed that the capsules formed around siCOLL1A1/MSN-PEI- and siCOLL1A1/TKO[®]-encapsulated scaffolds were significantly thinner after 2 and 4 weeks as compared to siNEG/MSN-PEI-encapsulated scaffolds and untreated scaffolds (Plain). After 2 weeks, the capsule reduction around siCOLL1A1/MSN-PEI-encapsulated scaffolds was significantly higher than siCOLL1A1/TKO[®]-encapsulated scaffolds (42.3 % and 23.0 % respectively). The capsule reduction obtained around siCOLL1A1/MSN-PEI-encapsulated scaffolds was

1 stable after 4 weeks. However, there was no prominent difference between
2 siCOLL1A1/MSN-PEI- and siCOLL1A1/TKO® -encapsulated scaffolds as the capsule
3 reduction was 45.8 % and 42.3 % respectively.

4. Discussion

8 Mesoporous silica nanoparticles have received increasing attention as a drug
9 delivery vehicle. While many studies have shown the efficacy of bolus-delivered MSNs
10 in drug release or as a vector for siRNA cellular uptake [13,14,18,20–22], there are no
11 studies that describe the release of siRNA/MSN complexes that is mediated by
12 scaffolds. To establish scaffold-mediated sustained delivery of siRNA/MSN complexes,
13 we have designed novel siRNA/MSN-PEI coated-scaffolds by fiber surface absorption
14 using mussel-inspired bioadhesive and siRNA/MSN-PEI encapsulated-scaffolds. As a
15 proof of concept, we investigated the efficacy of siRNA/MSN-PEI complexes in
16 controlling fibrous capsule formation to improve host-implant integration through the
17 down regulation of collagen type I (Coll1A1) *in vitro* and *in vivo*.

19 As a reductively cleavable linkage which is stable under physiological conditions
20 but will quickly degrade in a highly reductive environment in cells, the disulfide bond has
21 emerged as a biocompatible group for on-demand intracellular drug delivery.[38–40] To
22 facilitate the intracellular release of siRNA from the MSN vector after endocytosis,
23 reduction-responsive MSNs were prepared by co-hydrolyzation and co-condensation of
24 TEOS and bio-reducible BTES. Polyethylene imine (PEI) was grafted onto the surface of

1 the particles to create a homogeneous coating to adsorb the negatively charged siRNA.
2 As a start to test gene silencing with siRNA/MSN-PEI complexes, different complexation
3 mass ratios were analyzed. Although the minimum ratio that was required to fully
4 complex all siRNA was 1:20, optimal cellular uptake was obtained at a mass ratio of
5 1:40 via bolus transfection and scaffold-mediated delivery. This trend was also relatable
6 to gene silencing efficiency.

7
8 siRNA/MSN-PEI were successfully adsorbed on DOPA-coated fibers with a
9 loading efficiency of more than 90 %, similar to what was described with other
10 transfection reagents.[31] Overall, 90 % of siRNA coated on siRNA/TKO and
11 siRNA/MSN-PEI scaffolds were released within 30 days. However, siRNA release from
12 siRNA/MSN-PEI (1:40)-coated scaffolds and siRNA/TKO[®]-coated scaffolds was
13 delayed for 2 days. This could be explained by the higher zeta potential of the
14 complexes, which in turn increased the electrostatic interactions with the negatively
15 charged DOPA that coated the fiber scaffolds.[31]

16
17 On the other hand, randomly oriented nanofibers that encapsulated siRNA/MSN-
18 PEI complexes were successfully obtained by electrospinning. The encapsulation
19 efficiency was only around 80 % largely due to wastage during the electrospinning
20 process as described in other studies.[23–25] We showed that after a similar slight early
21 burst release, already reported in the literature [41–44], siRNA released from
22 siRNA/MSN-PEI-encapsulated scaffolds was significantly slower than from siRNA/
23 TKO[®]-encapsulated scaffolds. The differences in release kinetics might be due to the

1 smaller size of siRNA/TKO[®] complexes.[31] Nonetheless, the scaffold biological
2 performance (Figure 8) suggested that the siRNA released from the siRNA/MSN-PEI
3 and siRNA/TKO encapsulated-scaffolds were stable and biofunctional for at least 4
4 weeks *in vivo*.

5
6 The uptake efficiency was higher when cells were seeded on siRNA/MSN-PEI-
7 coated scaffolds as compared to conventional bolus delivery although smaller amounts
8 of siRNA was delivered by coated scaffolds as indicated by the release profile (76 nM
9 was released from 2 µg siRNA/MSN-PEI (1:40)-coated scaffolds at Day 4 vs. 100 nM
10 per well by bolus delivery). This could be attributed to the local availability of the
11 complexes at the surface of the scaffold where cells could easily uptake these drugs. To
12 improve the RT-PCR signal quality in silencing studies, the quantity of cells seeded was
13 higher (for the same amount of drug used) as compared to uptake studies. Despite an
14 increase in cell/siRNA ratio, significant protein silencing was obtained. Accordingly, 93.0
15 and 80.8 % of silencing efficiencies were reached on siRNA/MSN-PEI (1:40)-coated
16 scaffolds after 4 days when targeting Coll1A1 and CyB respectively. It has been shown
17 that although the siRNA release profile of coated-scaffolds stopped progressing after 3-
18 5 days *in vitro*, the gene silencing was sustained for at least 7 days.[32] This suggests
19 that the internalized RNAs came not only from the RNAs that were released by the
20 scaffolds but also from those that were captured by the seeded cells directly from the
21 scaffold. Consequently, such scaffolds could lead to sustained gene silencing
22 outcomes. Surprisingly, contrary to the conventional bolus delivery of siCOLL1A/TKO
23 complexes, siCOLL1A/TKO[®]-coated scaffolds showed a weaker silencing efficiency.

1 This could be explained by the two days lag time in the release profile and by the lower
2 uptake of siCOLL1A/TKO complexes by HDFs.

3
4 The slow release of siRNA from the encapsulated scaffolds led to a significantly
5 ($p < 0.05$) lower uptake rate as compared to bolus delivery (2.2 nM, 2.5 nM and 6.4 nM
6 from siRNA/MSN-PEI (1:40)-encapsulated scaffolds after 3, 7 and 14 days respectively
7 vs. 100 nM and 50 nM per well by bolus delivery). Nevertheless, the quantity of
8 siRNA/MSN-PEI complexes transfected in HDFs increased with the incubation time,
9 which resulted in a significant Coll1A1 and CyB silencing after 7 and 14 days. It was
10 possible that this delay corresponded to the time required to reach the effective
11 concentration threshold through siRNA accumulation. It has been shown previously that
12 electrospun PCLEEP fibers did not lose its molecular weight even after 2 months of
13 incubation in distilled water at 37°C. [28] This suggested that no degradation occurred
14 during the collagen knockdown experiment. After 5 months, the released siRNA
15 remained mostly complexed to MSN-PEI suggesting that siRNA/MSN-PEI complexes
16 were stable when encapsulated within the fibers. Interestingly, our results confirmed an
17 intrinsic collagen 1A1 silencing capability of the MSN-PEI, as shown previously by
18 Morry et al.[45] while no effect on cyclophilin B was seen. Morry et al demonstrated that
19 MSN possessed efficient antioxidant properties that led to the reduction of the
20 associated fibrotic markers such as collagen.[45] MSN-PEI are, therefore, of high
21 interest for the control of collagen expression.

1 In our study, we compared the use of degradable MSN-PEI to commercially
2 available TKO[®] for intracellular delivery of siRNA. Overall, MSN-PEI is a more robust
3 vector for intracellular drug delivery due to its high siRNA loading efficiency. As shown
4 in the Cy5-RNA uptake study, the amount of siRNA that was taken up by HDFs was
5 indeed higher when mediated by MSN-PEI as compared to TKO[®], both in bolus and
6 scaffold mediated-delivery. Despite a higher uptake efficiency, the *in vitro* silencing
7 efficiency of siRNA/MSN-PEI complexes was comparable to siRNA/TKO[®] complexes.
8 As a comparison, we have previously used cell penetrating peptides, MPG and CADY,
9 as siRNA delivery vectors to silence collagen 1A1 *in vitro*. [25] There, we showed that
10 the collagen silencing efficiency of siCOLL1A1/MPG and siCOLL1A1/CADY through
11 bolus delivery was 3 and 4 time less efficient as compared to siCOLL1A1/TKO[®]
12 complexes.

13
14 The *in vivo* biodistribution study showed that the Cy5-RNA diffusion was visible
15 starting 7 days post implantation and extended up to at least 14 days. This result could
16 be correlated with *in vitro* silencing results since the silencing efficiency began after 7
17 days and was increased after 14 days. After 2 weeks, despite that a lower amount of
18 siRNA that was released from siCOLL1A1/MSN-PEI-encapsulated scaffolds as
19 compared to siCOLL1A1/TKO[®]-encapsulated scaffolds (22.2 % vs. 65.7 %
20 respectively), siCOLL1A1/MSN-PEI-encapsulated scaffolds led to a higher *in vivo*
21 capsule reduction (42.3 % vs. 23.0 % respectively). However, after 4 weeks, although
22 the differences in the siRNA amount released remained prominent (32.1 % vs. 67.3 %
23 for siCOLL1A1/MSN-PEI-encapsulated and siCOLL1A1/TKO[®]-encapsulated scaffolds

respectively), the *in vivo* capsule reduction was similar around both scaffolds. This trend might be explained by the natural wound healing process where the fibrous capsule would continue to compact and reduce in thickness with prolonged periods of recovery.[46] In addition, the higher MSN silencing efficiency that was observed *in vivo* could be correlated with the higher *in vitro* collagen silencing that was obtained with siRNA/MSN complexes as compare to TKO after 3 and 7 days (Figure 6). Hence, our *in vitro* and *in vivo* results suggest the benefits of using MSN-PEI as a siRNA delivery vehicle.

Taken together, scaffold mediated delivery is a promising approach to achieve sustained, non-viral delivery of siRNA. Future applications in tissue engineering and regenerative medicine could be envisaged. For example, the combined delivery of multiple siRNAs within the same scaffold, with one encapsulated, and the other surface coated, could allow dual or sequential drug release profiles to be obtained.

5. Conclusion:

In this work, we designed nanofiber scaffolds for localized and sustained delivery of siRNA/MSN-PEI complexes. Specifically, these complexes were either incorporated onto the surfaces of nanofiber substrates or directly encapsulated within nanofibers. Such scaffolds provided sustained availability of siRNA for at least 30 days (siRNA/MSN-PEI-coated scaffolds) and 5 months (siRNA/MSN-PEI-encapsulated scaffolds) *in vitro*. The biofunctionality of the scaffolds were further demonstrated *in vitro* and *in vivo* by targeting COL1A1 as the model protein to decrease fibrous capsule

formation to improve host-implant integration. Since MSN-PEI are also ideal for the delivery of low molecular weight drugs, this platform may also extend to future dual or sequential drug/gene delivery applications for directing cell fate and tissue regeneration.

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The bolus delivery of siRNA/ Mesoporous Silica Nanoparticles (MSN) complexes shows high efficiency to silence protein agonists of tumoral processes as cancer treatments. However, in tissue engineering area, scaffold mediated delivery is desired to achieve a local and sustained release of therapeutics. We showed the feasibility and the efficacy of siRNA/MSN delivered from electrospun scaffolds through surface adsorption and nanofiber encapsulation. We showed that this method enhances siRNA transfection efficiency and sustained targeted proteins silencing *in vitro* and *in vivo*. As a proof of concept, in this study, we targeted collagen type I expression to modulate fibrous capsule formation. However this platform can be applied to the release and transfection of siRNA or miRNA in cancer and tissue engineering applications.

COVER LETTER

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11th May 2018

Professor W. R. Wagner
Editor-in-Chief
Acta Biomaterialia

Dear Professor W. R. Wagner

Attached, please find our revised manuscript, **"Sustained delivery of siRNA/mesoporous silica nanoparticle complexes from fiber scaffolds for long-term gene silencing"**, submitted for consideration of publication in *Acta Biomaterialia* together with our detailed responses to reviewer's comments.

In this work, we incorporated siRNA/MSN complexes into electrospun fiber scaffolds. We evaluated the efficacy of these constructs in enhancing host-implant integration by decreasing fibrous capsule formation using RNA interference. Two incorporation methods were considered: fiber surface absorption using mussel-inspired bioadhesive and direct encapsulation within nanofibers. Our results show that scaffolds provided controllable, localized and sustained release of siRNA/MSN. As compared to conventional bolus delivery *in vitro*, such scaffold-mediated transfection provided more effective gene silencing. Additionally, *in vivo* subcutaneous implantation revealed localized delivery of siRNA and a significant reduction in fibrous capsule formation. These findings clearly demonstrate the advantage of our siRNA/MSN-incorporated scaffolds for sustained non-viral gene silencing. This manuscript should be of interest to readers in the field of biomaterials, drug/gene delivery and regenerative medicine.

Thank you for your kind consideration of this manuscript.

Sincerely,

A handwritten signature in black ink, appearing to read "Sing Yian Chew".

Sing Yian Chew, Ph.D.

Captions.

Table 1. siRNA and delivery vector complexation ratios, *in vivo* time points and sample numbers per group for *in vivo* biodistribution and gene silencing studies.

Table 2. Characterization of siRNA/MSN complexes and scaffolds incorporation parameters.

Figure 1. Degradable MSN-PEI: (A) Representative schemes of degradable MSN-PEI, (B) TEM image of MSN-PEI, (C) TEM image of degradation product of MSN-PEI after 72h in Dithiothreitol.

Figure 2. Sustained delivery of siRNA/MSN-PEI complexes: From (1:40) siRNA/MSN-coated (A-C) and - (1:40) encapsulated scaffolds (D-F). (A, D) Cy5-RNA homogenous distribution on scaffolds, (B, E) SEM images for the scaffolds after complexation with siRNA/MSN and before release, the arrows show the scaffolds siRNA/MSN-PEI coating (C, F) SEM images for the scaffolds after siRNA/MSN release kinetics, (G, H) *in vitro* siRNA release kinetics in PBS at 37°C and (I) Graph showing that siRNA released from siRNA/MSN-encapsulated scaffolds after 33 and 150 days was still complexed with MSN as compared after heparin decomplexation.

Figure 3. Cellular uptake of Cy5-RNA/MSN-PEI complexes by HDF. (A) Representative fluorescent microscopy images of HDFs after 3 days bolus transfection with 50 nM and 100 nM of Cy5-RNA complexed with MSN-PEI at different mass ratios (1:20 and 1:40); and TKO. Scale bar= 50 μ m. (B) Representative fluorescent microscopy images of cells after 4 days of culture on scaffolds coated with 2 μ g and 4 μ g of Cy5-RNA complexed with MSN-PEI at different mass ratios (1:20 and 1:40); and TKO. Scale bar = 25 μ m. (C) Representative confocal microscopy images of cells after 3, 7 and 14 days on scaffolds encapsulated with Cy5-RNA/MSN-PEI complexes. Scale bar = 10 μ m. (D) Quantification of the Cy5 signal intensity, * $p < 0.05$ and ** $p < 0.1$

Figure 4. *In vitro* silencing efficacy of bolus delivery siCOLL1A1/MSN-PEI complexes: Collagen 1A1 mRNA expression evaluation demonstrated effective gene silencing 3 days after siCOLL1A1/MSN-PEI delivery. * $p < 0.05$.

Figure 5. *In vitro* silencing efficacy of siRNA/MSN-PEI - coated scaffolds: (A) Collagen 1A1 mRNA expression evaluation demonstrated effective gene silencing 4 days after cell seeding on siCOLL1A/MSN-PEI scaffolds, * $p < 0.05$. Plain controls: untreated scaffolds (B) Immunofluorescence images of HDFs revealing the silencing of Collagen 1A1 on siRNA/MSN-PEI-coated scaffolds (Green: collagen; blue: nuclei), scale bar= 20 μ m, (C) Cyclophilin B mRNA expression evaluation demonstrated effective gene silencing 4 days after cell

seeding on sicyB/MSN-PEI scaffolds. * $p < 0.05$. Plain controls: untreated scaffolds

Figure 6. *In vitro* silencing efficacy of siRNA/MSN-PEI - encapsulated scaffolds: (A) Collagen 1A1 mRNA expression evaluation demonstrated effective gene silencing at 3,7 and 14 days after cell seeding on siCOLL1A/MSN-PEI scaffolds. * $p < 0.05$, Plain controls: untreated scaffolds (B) Immunofluorescence images of HDFs revealing the silencing of Collagen 1A1 on siRNA/MSN-PEI-encapsulated scaffolds (Green: collagen; blue: nuclei), scale bar: 20 μm . (C) CyB mRNA expression evaluation demonstrated effective gene silencing at 3,7 and 14 days after cells seeding on sicyB/MSN-PEI scaffolds. * $p < 0.05$, ** $p < 0.1$. Plain controls; untreated scaffolds.

Figure 7. *In vivo* biodistribution of Cy5-RNA released from Cy5-RNA/MSN-PEI-encapsulated scaffolds after 3, 7 and 14 days post implantation: (Left) Bright field image illustrating scaffold/tissue interface (white dotted lines outline interface). Scale bar: 100 μm (Right) Confocal images showing Cy5 fluorescence signal diffusion in tissues. White arrows: Cy5 fluorescent signals. Scale bar= 100 μm

Figure 8. (A) Histological images showing the fibrous capsule that encapsulated the nanofiber scaffolds after 2 and 4 weeks of implantation. Masson Trichrome staining (left) and Hematoxylin& eosin staining (right). The distance between the full arrowheads indicates the thickness of the fibrous capsule and the dotted

arrows show the part of the scaffold in contact with the capsule. (B) Quantification of the capsule thickness. * $p < 0.05$, % and # compared to plain and siNEG/MSN-PEI at week 2, $p < 0.05$, + and § compared to plain and siNEG/MSN-PEI at week 4, $p < 0.05$, scale bar = 50 μm . Plain controls: untreated scaffolds

Figure 1
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Table 1

Study	Samples	Ratio	unit	Time point	no. of samples
Biodistribution	cy5-ODN/MSN-PEI	1:40	w/w	3 d	3
				1 wk	3
				2 wk	3
<i>In vivo</i> silencing	CONTROL	-	-	2 wk	4
				4 wk	4
	siNEG /MSN-PEI	1:40	w/w	2 wk	4
				4 wk	4
	siCOLL1A /MSN-PEI	1:40	w/w	2 wk	4
				4 wk	4
	siCOLL1A/TKO®	1:1	v/v	2 wk	4
				4 wk	4

Table 2

Samples	Ratio	unit	Complexes size	Zeta potential	Incorporation methods	siRNA (mass)
MSN	-	-	132 ± 1 nm	54 ± 0.9 mV	-	-
siNEG/MSN-PEI	1:20	w/w	705 ± 24 nm	16.3 ± 2.9 mV	• Coated-scaffold	2 and 4 µg
siNEG/MSN-PEI	1:40	w/w	588 ± 16 nm	20.5 ± 2.2 mV	• Coated-scaffold • Encapsulated -scaffold	2 and 4 µg 11 µg

Supplementary Material

[Click here to download Supplementary Material: PINESE et al Supplementary data.pdf](#)