Modulating macrophage phenotype by sustained microRNA delivery improves host-implant integration

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Title: Modulating macrophage phenotype by sustained microRNA delivery improves host-implant integration

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Abstract

Biomedical implant failure due to the host's response remains a challenging problem. In particular, the formation of the fibrous capsule is a common barrier for the normal function of implants. Currently, there is mounting evidence indicating that the polarization state of macrophages plays an important role in effecting the foreign body reaction (FBR). This opens up a potential avenue for improving host-implant integration. Here, we utilized electrospun poly(caprolactone-co-ethyl ethylene phosphate) nanofiber scaffolds to deliver microRNAs (miRs) to induce macrophage polarization and modulate FBR. Specifically, C57BL/6 mice that were treated with M2-inducing miRs, Let-7c and miR-124, displayed relatively thinner fibrous capsule formation around the scaffolds at both Week 2 and 4, as compared to treatment with M1-inducing miR, Anti-Let-7c. Histological analysis showed that the density of blood vessels in the scaffolds were the highest in miR-124 treatment group, followed by Anti-Let-7c and Let-7c treatment groups. Based on immunohistochemical quantifications, these miR-encapsulated nanofiber scaffolds were useful for localized and sustained delivery of functional miRs and were able to modulate macrophage polarization during the first 2 weeks of implantation to result in significant alteration in host-implant integration at longer time points.

1. Introduction

Foreign body reaction (FBR) towards surgically implanted biomaterials, prostheses or medical devices is a multistage host's response.^[1] This process is initiated when an implant

comes into contact with blood and usually ends with a layer of dense collagenous deposition that surrounds the implant, a phenomenon known as fibrous encapsulation. The formation of these collagenous structures is an evidence of wound healing and has been recognized to be a consequence of the reciprocal interactions between macrophages and fibroblasts.^[2] Although it is perceived by the body to be beneficial, encapsulation of the implant will isolate it from the surrounding tissues,^[3] thereby hindering its intended functions, especially for drug delivery systems.^[4] For the past few decades, efforts to prevent implant fibrosis have varied and encompassed the alteration of material properties (porosity, topography, shape, surface chemistry)^[5-8] and the regulation of cellular aspects such as collagen production in fibroblasts.^[9-11] Unfortunately, despite these endeavours, success remains limited due to the complexity of this physiological mechanism.

Currently, there is a general consensus that macrophages play a critical and diverse role in mediating FBR and dictating implantation outcomes^[12] due to their persistent presence and reactivity to the microenvironment. Macrophages are dynamic and can adopt distinct functional phenotypes within a spectrum (M1 and M2). The acquisition of these phenotypes, otherwise known as polarization, occurs depending on the factors to which macrophages are being exposed.^[13] Apart from conventional signalling molecules (e.g. LPS, IL-4, etc), the use of RNA interference (RNAi), such as microRNAs (miRs), is also a good alternative for inducing macrophage polarization.^[14] MiRs are small, non-coding RNA molecules that are involved in gene and protein regulation^[15] and have found useful applications in cancer diagnosis and treatment.^[16] One of the main advantages presented by miRs over conventional signalling molecules is their ability to target multiple genes and pathways.^[17] Correspondingly, they could potentially elicit more pronounced biological effects.

71
72 Recently, Banerjee *et al.* identified Let-7c and its corresponding miR inhibitor, Anti-
73 Let-7c to be involved in regulating macrophage polarization.^[18] In particular, the
74 overexpression of Let-7c resulted in mouse bone marrow-derived macrophages adopting a
75 more M2 phenotype, that was characterized by the gene expression of Arg1, FIZZ1 and YM-1.
76 On the other hand, the inhibition of Let-7c led to macrophages possessing a more M1
77 phenotype, which was indicated by the increased gene expression of IL-12 and iNOS.^[18]
78 Similar to this work, Ponomarev *et al.* identified another miR, miR-124, to be implicated in
79 skewing macrophages' activation towards the M2 phenotype.^[19] While both studies provided
80 comprehensive information on the mechanics of the signalling pathways, there was a lack of
81 therapeutic applications of these miRs.

82
83 In this study, we explore the feasibility of delivering miRs by electrospun scaffolds to
84 modulate macrophage phenotype and improve host-implant integration. Specifically, we
85 fabricated a scaffold that comprised of randomly-oriented poly(caprolactone-co-ethyl
86 ethylene phosphate) (PCLEEP) nanofibers, which encapsulated miRs. Nanofiber scaffolds
87 were the preferred choice due to their architecture, which mimics the extracellular matrix
88 (ECM). Moreover, their topographical orientation were also shown to affect fibrous capsule
89 formation.^[20] Furthermore, the versatile electrospinning process allows it to serve as a
90 surface coating method to modify surfaces and improve tissue integration of other implants,
91 such as neural electrodes^[21] and orthopaedic implants.^[22] We showed that electrospun
92 scaffolds are well-suited for delivering small non-coding RNAs, such as miRs and small
93 interfering RNAs (siRNAs) both *in vitro*^[23-26] and *in vivo*.^[9, 27] Here, we hypothesize that

nanofibers that are endowed with miRs, which can modulate macrophage phenotype, may potentially ameliorate fibrous capsule formation and enhance implant acceptance by the host.

2. Results

2.1. RNA-loaded electrospun fibers were successfully fabricated

To evaluate the drug distribution of miR throughout the scaffold, AF488-RNA, which consisted of similar number of base pairs as miR, was encapsulated within the nanofibers using electrospinning, as shown in **Figure 1A**, 1B and 1C. In addition to the uniform distribution of miR throughout the scaffolds, bead-free miR-encapsulated fibers were also observed (Figure 1D). The average diameters of the scaffolds remained similar regardless of drug encapsulation, as shown in **Table 1**.

The encapsulated miRs within each 1 x 2 cm piece of scaffold were released in a sustained manner for at least 30 days after an initial burst release upon immersion into PBS (Figure 1E). A total of 52.5% of miRs were released over the span of 30 days, of which around 84.5% were released between Day 0 and 7. Thereafter, the remaining 15.5 % accounted for the sustained release up to Day 30. The experimental loading efficiency of miRs into electrospun PCLEEP scaffolds was 26.4 ± 7.3 %.

2.2. Mice implanted with scaffolds containing M2 macrophage-inducing miRs possessed thinner fibrous encapsulate

The fibrous capsule was defined as the dense and relatively acellular collagenous structure that surrounded the scaffold implant (collagen stains blue in Masson's trichrome staining)(**Figure S1**). Since the regions around the scaffolds also consisted of endogenous

collagen, EVG stained images (**Figure 2A and 2B**) were also used to confirm the identification of the fibrous capsule based on the absence of elastin (elastin stains black in EVG staining). As seen from the Masson's trichrome stained images, scaffolds that encapsulated Let-7c, an M2-inducing miR, led to thinner fibrous encapsulation ($10 \pm 3.5 \mu\text{m}$ at Week 2 and $11.5 \pm 1.6 \mu\text{m}$ at Week 4) as compared to Anti-Let-7c, an M1-inducing miR ($17 \pm 4.5 \mu\text{m}$ for Week 2 and $21 \pm 4.3 \mu\text{m}$ for Week 4)(Figure 2A to 2C). To confirm the effects of M2-inducing miR on fibrous capsule formation, we also included another previously reported M2-inducing miR, miR-124, in our study. Similar trends of reduction in capsule thickness were obtained for miR-124 treatment (Figure 2A to 2C; $7.4 \pm 2.9 \mu\text{m}$ at Week 2 and $7 \pm 1.3 \mu\text{m}$ at Week 4). For the control groups, plain scaffolds resulted in a relatively thin capsule ($13.8 \pm 4.4 \mu\text{m}$) at Week 2 (Figure 2A and 2C). However at Week 4 (Figure 2B and 2C), the capsule increased in thickness ($14.6 \pm 3.8 \mu\text{m}$) and was comparable to the measurement from Neg-miR encapsulated scaffolds (Figure 2B and 2C) ($14.6 \pm 4.7 \mu\text{m}$). Overall, statistical analysis showed that there was a significant difference between Anti-Let-7c and miR-124 treatment groups at Week 4 ($p < 0.05$)(Figure 2C).

2.3. Scaffolds containing miR-124 showed more extensive blood vessel formation

The density of blood vessels in plain scaffolds remained rather consistent at both Week 2 (59.4 ± 67.7 blood vessels mm^{-2}) and Week 4 (34.3 ± 35.4 blood vessels mm^{-2}) (**Figure 3A and 3B**) as compared to scaffolds that encapsulated M2-inducing Let-7c, where the highest number of blood vessels per area were observed at Week 2 (87.2 ± 63.4 blood vessels mm^{-2}) (Figure 3A and 3B). However, by Week 4, Let-7c treatment resulted in the lowest blood vessel density (35.9 ± 28.8 blood vessels mm^{-2}) relative to all the other treatment groups (Figure 3A and 3B). Neg-miR loaded scaffolds had a slight decline in blood vessel density as well from

Week 2 (78.8 ± 24.1 blood vessels mm^{-2}) to Week 4 (57.8 ± 8.6 blood vessels mm^{-2}) (Figure 3A and 3B). Conversely, there was a modest increase in blood vessel density with time in M1-inducing Anti-Let-7c group at Week 2 (59.5 ± 60.4 blood vessels mm^{-2}) vs. Week 4 (71.8 ± 14 blood vessels mm^{-2}) (Figure 3A and 3B). In miR-124 samples, although it had the smallest blood vessel density at Week 2 (36.4 ± 22.7 blood vessels mm^{-2}), there was a three-fold increase at Week 4 (101.4 ± 20.4 blood vessels mm^{-2}) (Figure 3A and 3B) and was the highest as compared to the rest of the treatment groups. In addition, statistical analysis showed that between the treatment groups at Week 4, miR-124 was significantly different ($p < 0.05$)(Figure 3B) from both plain and Let-7c samples. MiR-124 treatment at Week 4 was also statistically different from miR-124 treatment at Week 2 ($p < 0.05$)(Figure 3B). Of note, there was a clear indication that Anti-Let-7c and miR-124-encapsulated scaffolds contained the largest average blood vessel size at Week 2 ($135.9 \pm 158.7 \mu\text{m}^2$ and $103.6 \pm 105.4 \mu\text{m}^2$ respectively) and Week 4 ($108.7 \pm 127.8 \mu\text{m}^2$ and $130 \pm 131.8 \mu\text{m}^2$ respectively) (Figure 3C).

2.4. Macrophage expression is associative with fibrous capsule thickness at Week 2

Macrophage phenotype was determined via the coexpression of the pan-macrophage marker, F4/80, with the M1 marker, iNOS, or M2 marker, CD206. At Week 2, macrophages adopted expressions that closely corresponded to the treatment administered, suggesting that the miRs were indeed able to polarize the macrophages (Figure 4A, 4B and 4C). Specifically, the M1 macrophage-inducing miR, Anti-Let-7c, led to more iNOS-expressing macrophages within and outside the scaffolds (Figure 4A, 4B and 4C) while the M2 macrophage-inducing miRs, Let-7c and miR-124, led to more CD206-expressing macrophages within and outside the scaffolds (Figure 4A, 4B and 4C). When we compared the correlation between fibrous encapsulate thickness and macrophage phenotype outside the scaffold,

there were more iNOS-expressing macrophages present in the vicinity of scaffolds containing thick fibrous encapsulation (Neg-miR and Anti-Let-7c). Conversely, scaffolds with relatively thinner fibrous capsule were surrounded with CD206-expressing macrophages instead (Let-7c and miR-124)(Table S1A).

2.5. Macrophage expression is not associative with fibrous capsule thickness at Week 4

Compared to the macrophage expression profile at Week 2, the macrophages within and outside the scaffolds at Week 4 generally expressed more iNOS, with the exception of macrophages found outside Let-7c loaded scaffolds (Figure 5A, 5B and 5C). In particular, the macrophages that were outside miR-124-loaded scaffolds expressed more iNOS as compared to the macrophages that resided outside the other scaffolds (Figure 5A, 5B and 5C). Despite that, the fibrous layer surrounding the miR-124 loaded scaffold at Week 4 was the thinnest (Figure 2C). Altogether, the results at Week 4 did not appear to maintain the trend that was observed in Week 2. Specifically, the abundance of iNOS signals at Week 4 did not correspond to the formation of thicker fibrous encapsulate and the enhancement of CD206 signals did not result in thinner fibrous encapsulate (Figure S1B).

3. Discussion

Foreign body reaction (FBR) remains a significant problem for biomedical implants. The formation of dense collagenous structures not only physically isolates the implant from the host tissues but also limits the diffusivity of loaded drugs, resulting in hindered treatments. A logical approach to retain the functionality of the implant would, thus, encompass imparting it with the ability to mitigate FBR. We demonstrated that nanofibers are better than films in

reducing implant fibrosis^[20] and when loaded with siRNA against collagen type 1 (siCOL1A1), fibrous capsule formation was reduced further.^[9, 11] Nanofiber scaffolds are biomimicking due to their structural similarity to the extracellular matrix.^[28-30] Furthermore, they provide topographical cues to direct cell fate.^[28, 31] Besides being applicable as an implantable device by itself, electrospun scaffolds can also be used to coat other biomedical devices to enhance the overall implant biocompatibility.^[21, 22] Han N. and co-workers showed that nanofibers, when introduced by the versatile electrospinning technique, could also be adapted as surface modifications on neural prostheses to moderate FBR.^[21] Apart from providing biomimicking architecture, we have demonstrated sustained non-viral delivery of small non-coding RNAs (e.g. siRNAs) using nanofiber platform both *in vitro*^[24-27] and *in vivo*.^[9, 11, 27] In these studies, siRNAs were delivered with the intention of affecting a specific gene. However, since biological systems frequently contain many redundancies, the suppression of a single gene may not be sufficient to elicit significant changes in biological outcomes. Therefore, miRs, which could regulate several genes at once^[17] were explored in this work. Here, we targeted the macrophages as they have reciprocal interactions with fibroblasts and are also the main contributors to fibrous capsule formation. A system that targets the immune response can also have wider implications in the amelioration of other diseases that involve macrophages, such as cancer and atherosclerosis.^[32]

In this work, we established a platform that is able to deliver functional and novel miRs non-virally to modulate macrophage responses and enhance host-implant integration by decreasing fibrous capsule formation more effectively than our previous works.^[9, 11, 20]

Scaffolds were electrospun from PCLEEP, a synthetic polymer that has enhanced biodegradability as compared to PCL^[30] due to its hydrophilicity. The encapsulation of small

non-coding RNAs also served to protect these labile molecules from biodegradation while enabling long term gene silencing due to the sustained availability of the drugs. We showed that by encapsulating miRs within PCLEEP fibers, a sustained release of miRs could be achieved for at least 30 days (Figure 1E). Besides that, as miR dosage is an important determinant in achieving favourable biological responses,^[33, 34] the maximum amount of miR that could be loaded into each scaffold was utilized. Apart from the chemical cues from miRs, topographical signals, in the form of fiber diameter^[31, 35] and alignment,^[20] also impact cellular behaviour. Based on these studies, randomly-oriented PCLEEP nanofiber scaffolds, which elicited thinner fibrous capsule formation than its aligned counterpart, with similar fiber diameter range were fabricated for this work (Table 1). Using AF488-RNA as the model drug for ease of visualization, we could infer that these scaffolds contained a uniform distribution of miRs, which would allow cells that are in contact with the scaffolds to have equal accessibility to these biomolecules.

Of note, while the average scaffold fiber diameter between each group was not significantly different, they were also not perfectly similar. Therefore, there is a possibility that minute fiber diameter differences, in the range of tens of nanometers, may lead to intrinsic variations between scaffolds pertaining to miR delivery, macrophage polarization and blood vessel formation. To be certain that the results we observed were indeed miR-driven and not due to fiber diameter effects, we referred to the literature for validation. For miR delivery, we have previously loaded miRs onto electrospun fibers with various diameters and measured the release profiles.^[23] Even though the standard deviation of the fiber diameter exceeds tens of nanometers, the variation in the release of the miRs was less than 5%. Extrapolating from these results, we believe that the miR release profile would not be

significantly affected by the fiber diameter differences. In terms of macrophage polarization, Saino *et al.* demonstrated that macrophages secreted more proinflammatory cytokines when seeded on micro-sized fibers ($1.60 \pm 0.25 \mu\text{m}$) than on nano-sized fibers ($0.55 \pm 0.16 \mu\text{m}$).^[35] However, comparing the standard deviation of their nanofibers, which were $\sim 160 \text{ nm}$ and much larger than the tens of nanometers fiber diameter difference between our scaffolds, we believe that macrophage polarization would not be significantly altered by the scaffolds (without miRs) used in our study. Lastly, we did not manage to find any literature that investigated the effects of electrospun fiber diameter on blood vessel formation. However, based on the findings from the release profile and macrophage polarization, we believe that, without miRs, the scaffolds used in this study would not result in significant difference in blood vessel formation.

The miRs of interest in this study were initially Let-7c and anti-Let-7c as they had been reported to affect macrophage polarization. Specifically, the overexpression of Let-7c induced M2 macrophage polarization whereas anti-Let-7c promoted M1 macrophage polarization by inhibiting the function of endogenous Let-7c.^[18] However, we also included miR-124, another M2 macrophage inducing miR,^[19] in order to further validate our observed outcomes where the M2-inducing miR, Let-7c, led to thinner fibrous capsule formation. As we were more interested in confirming the trend involving the reduction of fibrous encapsulation, an additional M1-inducing miR was not used for this study. Currently, it has been well-documented that macrophages are key mediators of fibrosis based on their interactions with fibroblasts during an inflammatory response.^[36] Consequently, the regulation of the phenotypic state of macrophages may alter host-implant integration outcomes.^[1] The purpose of our study primarily revolves around managing fibrous capsule formation, which is

a measure of how well the implanted biomaterial can be accepted by the host. The time points chosen (Week 2 and Week 4) for evaluating fibrous capsule thickness were chosen based on a previous study by Brown *et al.*^[37] and our published works.^[9, 20] From the Masson's trichrome stained samples, we observed that scaffolds, which encapsulated Let-7c and miR-124, both M2-inducing miRs, resulted in thinner fibrous encapsulation as compared to the rest of the treatment and control groups throughout the duration of the implantation (up to 4 weeks). The opposite was true, where scaffolds containing Anti-Let-7c, an M1-inducing miR, had a relatively thicker fibrous capsule than the controls (plain and Neg-miR scaffolds at 4 weeks). This suggested a correlation between the effect of M1 and M2-inducing miRs and thickness of fibrous capsule formation. In order to establish this relationship, we proceeded to evaluate the phenotypic condition of the macrophages after being subjected to miR treatment.

Even though it is known that macrophages regulate foreign body reaction, there is currently no known exact macrophage polarization state that can lead to the most ideal biomaterial integration outcome. Attempting to solve this conundrum with a simple M1 or M2 macrophage skewing would also be undermining the complex processes that occur during this period of extensive ECM remodelling. Regardless, the use of both Let-7c and miR-124 appeared to result in thinner fibrous capsule formation. Taken together, these results seemed to imply that the initial macrophage response is able to determine the ultimate capsule thickness. Even though the expected macrophage polarization was not observed at the end of Week 4, the initial polarization at Week 2 appeared to be sufficient in altering the final thickness of the capsule (Figure 2). Additionally, sustained delivery of the miRs for tuning macrophage polarization might not be a necessary criteria for modulating fibrous capsule

thickness. However, more work is required to elucidate this apparent switch in the mechanism that regulates fibrosis. Future studies could include systematic gene screening of the macrophages that are present in miR-124 treated groups during Week 2 to Week 4 to identify potential factors that may reduce the extent of fibrous encapsulation and also utilize this platform as a drug depot for implantation to treat diseases. Furthermore, while the miRs were able to skew iNOS and CD206 expression in macrophages, future work should include more macrophage markers to provide a more accurate evaluation of their status following treatment *in vivo*. Additionally, cytokines, especially those that are produced locally and systemically by macrophages, may also be profiled to connect the miR treatments with pro- vs. anti-inflammatory responses.

Besides evaluating fibrous encapsulation, other measures of host-implant integration include the presence of blood vessels,^[38] as well as foreign body giant cells (FBGCs)^[39] around or within the scaffold. Generally, an increased quantity of large-sized blood vessels suggests enhanced integration. In this study, we utilized H & E staining, instead of CD31 immunofluorescent staining, for quantification because it was easier to identify functional blood vessels (blood vessels containing red blood cells). This method, however, is only recommended for formalin-fixed paraffin embedded (FFPE) and resin embedded samples since their tissue morphology are usually very well-preserved. Recent studies have reported that M2-polarized macrophages promoted angiogenesis^[40] as well as vasculogenesis.^[41] Yet on the other hand, there are studies that showed M1-polarized macrophages to be able to promote angiogenesis as well, secreting VEGF and stimulating endothelial cell sprout formation both *in vitro* and *in vivo*.^[42, 43] Here, we observed that blood vessels were more abundant in animals that had Anti-Let-7c and miR-124 treatment at Week 4. In Let-7c

treatment groups, a large number of blood vessels were found within the scaffolds at Week 2 instead. Besides blood vessel quantity, Anti-Let-7c and miR-124 samples also resulted in larger blood vessels being formed at both Week 2 and Week 4. Let-7c was able to decrease the extent of fibrous encapsulation as well but was not able to induce blood vessel formation. Lastly, although Anti-Let-7c was as potent as miR-124 in stimulating blood vessel growth, it fared the worst in regulating fibrosis. Taken together, miR-124 may be the preferred choice as compared to Let-7c and Anti-Let-7c for reducing fibrous capsule thickness and enhancing blood vessel formation. Although FBGCs were also used for evaluating host-implant integration, we did not quantify them as they were not prevalent in all groups (Figure 2 and 3).

Comparing the results from our study with previous related *in vivo* works, which quantified fibrous encapsulation,^[9, 20, 44-47] the combination of both electrospun fibers and miRs may be a useful strategy to modulate fibrous capsule formation. In these studies, the scaffolding systems used included electrospun nanofibers,^[9, 20] hydrogels,^[44, 45] chemically crosslinked polymers^[46] and decellularized extracellular matrix.^[47] Among these systems, the fibrous capsule quantified in the non-treated groups for electrospun fibers^[9, 20] were never observed to be thicker than 100 μm . However, in other platforms,^[44-47] the fibrous encapsulation were always greater than 100 μm . This implied that intrinsically, electrospun fibers alone might be less prone to FBR and thus might be a better alternative as compared to other systems for reducing fibrous capsule formation (although it might also be dependent on the species tested since most of these studies were conducted in either mice or rats). This observation is consistent with our results, where the plain and Neg-miR groups elicited relatively thin fibrous capsules at the end of the study ($14.6 \pm 3.8 \mu\text{m}$ and $14.6 \pm 4.7 \mu\text{m}$

respectively). With the addition of M2-inducing miRs, the thickness of the fibrous encapsulation dropped further ($11.5 \pm 1.6 \mu\text{m}$ in Let-7c treated samples and $7 \pm 1.3 \mu\text{m}$ in miR-124 treated samples (Figure 2)) and the percent reduction, when compared to the controls, amounted to approximately 21 to 52 %. These percentage reductions are comparable to a similar study conducted previously, where siRNA against siCOL1A1 was utilized for suppressing collagen secretion in fibroblast (25 to 57 % reduction when compared to the controls).^[9] Even so, the significance of using miRs appears to be greater since the thinnest fibrous capsule that was formed in the presence of siCOL1A1 was around $25 \mu\text{m}$ whereas in this study, miR-124 treated samples had an average fibrous thickness of only $7 \pm 1.3 \mu\text{m}$.

4. Conclusion

This study demonstrates the successful fabrication of a nanofiber platform for sustained, non-viral delivery of biologically active miRs that appeared to have an effect on macrophage polarization *in vivo*. The encapsulated miRs were released locally in a sustained manner for at least 30 days and led to phenotypic changes in macrophages at the initial time point (2 weeks) as well as a reduction in fibrous capsule formation. The pronounced variation in fibrous capsule and blood vessel formation between the different treatment groups further corroborated the functionality of the miRs delivered.

5. Methods and Materials

AlexaFluor488-tagged oligoribonucleotide (AF488-RNA) was purchased from Integrated DNA Technologies, USA. 2,2,2-trifluoroethanol (TFE), heparin and Tween-20 were obtained from Sigma, USA. TE buffer (pH 8.0) and PBS (pH 7.4) were purchased from 1st Base,

Singapore. TransIT-TKO (TKO) reagent was purchased from Mirusbio, USA. Negative miR (Neg-miR), Let-7c, Anti-Let-7c and miR-124 were purchased from Ambion, USA. RiboGreen reagent and Anti-Rat HRP (Catalog number: 31470) were purchased from Invitrogen, USA. Superfrost Plus glass slides and DAPI were purchased from Thermo Fisher Scientific, USA. Rat F4/80 (clone Cl:A3-1, Catalog Number: ab6640) and Rabbit CD206 primary antibodies (Catalog Number: ab64693) were obtained from Abcam, UK. Mouse iNOS/NOS Type II (iNOS) primary antibody (Catalog Number: 610328) was purchased from BD Biosciences, USA. Fluoromount-G mounting medium and citrate buffer were bought from eBioscience, USA. Anti-Rabbit HRP (Catalog number: K4003) and Anti-Mouse HRP (Catalog number: K4001) were procured from Agilent Technologies, USA. OPAL™ kit (contains TSA Cy3, TSA Cy5 and TSA FITC) was bought from PerkinElmer, USA.

5.1. Electrospinning of miR-encapsulated PCLEEP nanofibers

AF488-RNA, having a similar number of base pairs as miRs, was used to assess fiber morphology and oligonucleotide distribution following electrospinning and drug encapsulation. PCLEEP fibers ($M_w = 59,102 \text{ g mol}^{-1}$) were synthesized as described previously.^[30] Fibers encapsulating AF488-RNA complexed with TKO transfection reagent (AF488-RNA complexes) were fabricated using a working solution that was prepared in the following order. First, 0.5 g of PCLEEP was dissolved in 1.232 ml of 2,2,2-Trifluoroethanol (TFE). Concurrently, 38 μl of TE buffer (pH 8.0) was mixed with 90 μl of 50 μM AF488-RNA and 180 μl of TKO (1:2 v/v RNA:TKO ratio) and the mixture was incubated at room temperature for 15 min to form the complexes. The resulting AF488-RNA/TKO mixture was then added to the initial PCLEEP solution and mixed thoroughly. The resulting solution contains 32% (w/v) of PCLEEP and has a theoretical loading of 60 μg of RNA. A flow rate of 0.6 ml/h (KD Scientific,

USA) was utilized for all electrospinning processes. High DC voltage (Gamma High Voltage Research, USA) was applied to the polymer mixture at a positive voltage of 7-11.5 kV (the voltage was increased as time passed to stabilize the polymer jet stream). A rotating (230 rpm), negatively charged strip of aluminium foil (-4 kV) was used as the collector whereas a 22G needle was used as the spinneret. To obtain miR-encapsulated PCLEEP fibers, AF488-RNA was replaced with either Neg-miR, Let-7c, Anti-Let-7c or miR-124. The electrospun fibers were allowed to accumulate on the aluminium foil for 2 to 3 hours to form a scaffold strip that was sufficiently thick to prevent folding upon *in vivo* implantation.

5.2. Evaluation of scaffold morphology and miR distribution in fibers

A glass slide was held in front of the collector during the electrospinning process for 20 s to collect some AF488-RNA encapsulated PCLEEP fibers for visual evaluation of miR distribution using a fluorescence microscope (Olympus IX71) at 60x magnification. Fibers were also sputter coated with platinum for 60 s at 20 mA and analyzed under a scanning electron microscope (SEM; JOEL, JSM-6390LA, Japan) at an accelerating voltage of 10 kV. The average fiber diameter was then determined using ImageJ (NIH, USA) by measuring 100 fibers per sample from high magnification (5000x) SEM images.

5.3. Analysis of miR release kinetics

From each of the scaffold strip that encapsulated a different miR, (namely Neg-miR, Let-7c, Anti-Let-7c and miR-124), a 1 x 2 cm piece (average mass of 88.6 ± 3.7 mg) was cut out and used for the release kinetics study. The various 2 cm² pieces were completely submerged in 2 ml of PBS (pH 7.4) using Teflon rings and incubated at 37 °C in a 12-well plate. At fixed time points, 1 ml of supernatant was retrieved and replenished with 1 ml of fresh PBS (pH

7.4). For each of the 1 ml supernatant, 4 μ l of heparin (5 mg ml⁻¹) was added to de-complex the miR from the TKO reagent. The concentration of miR that was released into the supernatant was then quantified using RiboGreen assay following manufacturer's protocol. Fluorescence intensity measurements were then carried out using a microplate reader (Tecan, Infinite 200, Austria). The percent of cumulative release, based on experimental loading, was averaged using measurements from all 4 types of scaffolds (Neg-miR, Let-7c, Anti-Let-7c and miR-124). The experimental loading efficiency of miR was evaluated based on previous protocols^[26]. Briefly, Neg-miR, Let-7c, Anti-Let-7c and miR-124 encapsulated PCLEEP scaffolds (2 cm²) used for the release assay were each dissolved in 3 ml of chloroform at the end of the release kinetics study. Thereafter, 500 μ l of PBS (pH 7.4) was added and the aqueous phase was extracted. Similarly, the miRs within the extracted aqueous solution were decoupled from TKO, using 4 μ l heparin (stock concentration of 5 mg/ml), before running RiboGreen assay. The efficiency of miR extracted from the dissolved scaffold solution was also accounted for by doping PCLEEP solutions with a known amount of miRs/TKO complex, after which, the polymer solution was vacuum-dried overnight to remove the solvents. The dry polymer constructs containing miRs/TKO were then dissolved in 3 ml of chloroform and added with 500 μ l of PBS (pH 7.4). The aqueous phase was extracted and 4 μ l of heparin (stock concentration of 5 mg ml⁻¹) was used to de-complex miR from the TKO reagent. The concentration of miRs extracted was quantified as described above to obtain extraction efficiencies. The experimental loading efficiencies were then computed (Equation 1).

$$\text{Loading Efficiency} = \frac{\text{Total amount of miRNA released} + \text{Actual amount of miRNA extracted}}{\text{Experimental miRNA loading}} \times 100\% \quad (1)$$

5.4. Implantation of scaffolds

Five types of PCLEEP scaffolds, namely plain, Neg-miR, Let-7c, Anti-Let-7c and miR-124 were subcutaneously implanted into 10-12 weeks old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME). All mice were monitored for signs of toxicity, including significant weight change, grooming, irritability and respiratory rate. All animal procedures were performed in accordance with an Institutional Animal Care and Use Committee (IACUC) protocol approved by Duke University. The scaffolds were cut into discs of 8 mm diameter using a biopsy punch and UV irradiated for 30 min prior to implantation. Mice were anesthetized using gas anesthesia (O_2 , 2 L min^{-1} , isofluorane, 2%). After induction of anesthesia, the surgical site was shaved and sterilized. An 8 mm incision on the back of the mouse was made and a subcutaneous pocket was created at the incision by blunt dissection. A scaffold was then inserted into each pocket. The incision was closed with 4-0 monofilament absorbable suture and the mice were monitored until full recovery from anesthesia. For each time point, a total of three animals for each treatment group were used and each animal was implanted with only one scaffold.

5.5. Histological and immunohistochemical analysis

At 2 and 4 weeks post implantation, the implant specimen along with some of the surrounding tissues were removed and fixed in 10% neutral buffered formalin (NBF) for at least 24 hours. The samples were then cut into two halves and embedded in paraffin, sectioned (3 μm thickness) and mounted onto Superfrost Plus glass slides. For each animal, a total of 3 sections were obtained and mounted separately onto 3 glass slides in an adjacent fashion for histological comparisons after staining. One complete set of samples were sent for routine Elastic-Van Gieson (EVG) staining, while the other two sets were stained with Masson's Trichrome and Hematoxylin and Eosin (H & E). The thickness of fibrous capsule was

quantified using Masson's Trichrome stained samples and were further confirmed using EVG stained samples. Quantification was conducted using a brightfield stitched collage consisting of several 10x magnified images (Leica DMI8) of Masson's Trichrome stained samples. Each of the stitched images was then overlaid, using Adobe Photoshop CS6 (Adobe Systems, USA), with a smartgrid containing 8 equidistance divisions (Figure S1). Following that, measurements were taken at regions where the divisions intersected with the fibrous capsule using ImageJ (NIH, USA). The density and size of blood vessels within the scaffolds were evaluated with H & E stained images. Images were taken using a light microscope (Leica DMI8) at 40x magnification. A total of 8 images were taken along the entire length of the scaffold. Blood vessels can be identified as structures possessing a lumen with red blood cells within. The total number of blood vessels were quantified using the Cell Counter plugin (ImageJ, NIH) and used for comparison. The area of a blood vessel was quantified by tracing out its periphery and measuring it using ImageJ (NIH, USA).

Immunofluorescent staining using OPALTM was performed to characterize macrophage polarization in response to the different treatments. Following deparaffinization, the 3 μ m thick sections were placed in citrate buffer and microwaved for antigen retrieval. Following that, non-specific antibody binding was reduced by incubating the samples in a 10% goat serum blocking solution for 10 min at room temperature. Sections were then decanted without washing and incubated with primary antibodies at room temperature for 30 min. Primary antibodies against the pan-macrophage marker rat F4/80 (1:50 dilution), the M1 macrophage marker mouse iNOS (1:100 dilution) and the M2 macrophage marker rabbit CD206 (1:200 dilution) were used. These antibodies were chosen as they have been shown previously to be reliable and specific.^[37, 48, 49] Samples were then washed thrice with PBS

before secondary antibody incubation for 10 min at room temperature: anti-rat HRP (1:500 dilution), anti-mouse HRP (1:500 dilution) and anti-rabbit HRP (1:500 dilution). Lastly, TSA FITC (1:100 dilution) was added to anti-rat HRP, TSA Cy5 (1:100) dilution was added to anti-mouse HRP and TSA Cy3 (1:100 dilution) was added to anti-rabbit HRP. To ensure that the signals observed were true and specific, a control staining (with secondary antibodies but no primary antibodies) was evaluated as well. Nuclei were labelled with DAPI (1:1000 dilution) and slides were mounted with Fluoromount-G mounting medium.

Confocal images were obtained with LSM800 (Zeiss, Germany) at 40x magnification using the pan-macrophage marker F4/80 staining as the reference channel. Images were taken at the z-level where F4/80 staining was the most prominent. A total of 10 images along the entire length of the scaffold were procured for each sample (5 images at the implant interface and 5 images outside the implant interface). Quantification and analysis (blinded assessment) of M1 and M2 macrophages were conducted using TissueQuest analysis software. The calculation for the ratio of M1 to M2 macrophages, transformed using a log scale for more intuitive interpretation,^[37] is described in Equation 2.

$$M1:M2 \text{ cell ratio (log scale)} = \log_{10} \frac{\text{Number of DAPI}^+F4/80^+iNOS^+ \text{ cell}}{\text{Number of DAPI}^+F4/80^+CD206^+ \text{ cell}} \quad (2)$$

Values larger than 0 indicate a predominance of iNOS-expressing macrophages while values smaller than 0 indicate a predominance of CD206-expressing macrophages.

The relative expression of iNOS to CD206 per cell, transformed using a log scale, is described in Equation 3.

$$M1: M2 \text{ fold difference (log scale)} = \log_{10} \frac{\text{Total area of iNOS}^+ \text{ pixels in Cell X}}{\text{Total area of CD206}^+ \text{ pixels in Cell X}} \quad (3)$$

where X refers to all the cells detected by the TissueQuest software

Values larger than 0 indicate that the macrophage expressed more iNOS than CD206 while values smaller than 0 indicate that the macrophage expressed more CD206 than iNOS.

5.6. Statistical Analysis

Shapiro-Wilk test was used to test for data normality while Levene's test was used to test for homogeneity of variance. Thereafter, for comparisons between groups that had equal variances, the one-way analysis of variance (ANOVA) was used to determine statistical significance of different treatments within each time point while independent-samples t-test was used to evaluate statistical differences between similar treatments for different time points. The Tukey post-hoc test was conducted for one-way ANOVA. The Kruskal-Wallis test (two-tailed) followed by the Mann-Whitney post-hoc test were used for data with unequal variances or non-parametric distribution. All analyses, $n = 3$ for each group, were conducted using the SPSS software. A p-value < 0.05 was considered statistically significant. Error bar represents standard deviation (SD), unless otherwise stated.

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Conflict of interest

The authors declare no conflict of interest.

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Figure 1. RNA-loaded electrospun fibers were successfully fabricated. (A) Fluorescence image depicting uniform distribution of AF488-RNA throughout PCLEEP fibers. **(B)** Phase Contrast image of RNA-loaded PCLEEP fibers. **(C)** Merged image of A and B. **(D)** SEM image of miRNA-encapsulated fibers showing uniform fiber diameter, $\phi = 403 \pm 3$ nm. **(E)** Cumulative

release of miRNA/TKO complexes from electrospun PCLEEP fibers. Data are represented as mean $\pm$ SEM; n = 4.

Figure 2. Mice implanted with scaffolds containing M2 macrophage-inducing miRNAs possessed thinner fibrous encapsulate. Representative Masson's Trichrome and Elastic Van Gieson stained images of scaffold-tissue interface at (A) Week 2 and (B) Week 4 for determining fibrous capsule thickness. The pair of black arrows demarcate the thickness of

the fibrous capsule while the black asterisk represents the region outside the scaffold. **(C)** Bar graph comparing the thickness of fibrous capsules between the various treatment groups at Weeks 2 and 4. *: p-value < 0.05; One-way ANOVA; data are represented as mean $\pm$ SD; n = 3 animals per group.

Figure 3. Scaffolds containing miR-124 showed more extensive blood vessel formation. (A) Representative H & E images of blood vessels (black arrow) found within the scaffolds after Week 2 and Week 4. **(B)** Graph depicting the number of blood vessels per area. **(C)** Size of blood vessels formed in various treatment groups at Week 2 and Week 4. *: p-value < 0.05; One-way ANOVA; data are represented as mean $\pm$ SD; n = 3 animals per group.

Figure 4. Macrophage expression is associative with fibrous capsule thickness at Week 2.

(A) Representative images of macrophages present in and outside the implanted scaffolds.

(B) Graph showing the polarization ratio of macrophages found in and outside different scaffolds. **(C)** Heat map depicting the polarization ratio of all macrophages ($n \geq 110$ cells

counted for each animal) found in all the animals in and outside different scaffolds. Red and green colour denote a predominance of iNOS and CD206 expression by each cell respectively.

Each heat map bar represents one animal. Data are represented as mean $\pm$ SD; $n = 3$ animals per group.

Figure 5. Macrophage expression is not associative with fibrous capsule thickness at Week 4. (A) Representative images of macrophages present in and outside different scaffolds. **(B)** Graph showing the polarization ratio of macrophages found in and outside different scaffolds. **(C)** Heat map depicting the polarization ratio of all macrophages ($n \geq 269$ cells counted for each animal) found in all the animals in and outside different scaffolds. Red and green colour denote a predominance of iNOS and CD206 expression by each cell respectively. Each heat map bar represents one animal. Data are represented as mean $\pm$ SD; $n = 3$ animals per group.

Table 1. Average fiber diameter of the various treatment groups

Treatment Groups	Fiber Diameter (Average $\pm$ SD; nm)
Plain	391 $\pm$ 7
Neg-miR	412 $\pm$ 9
Let-7c	426 $\pm$ 9
Anti-Let-7c	398 $\pm$ 6
miR-124	386 $\pm$ 5