ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

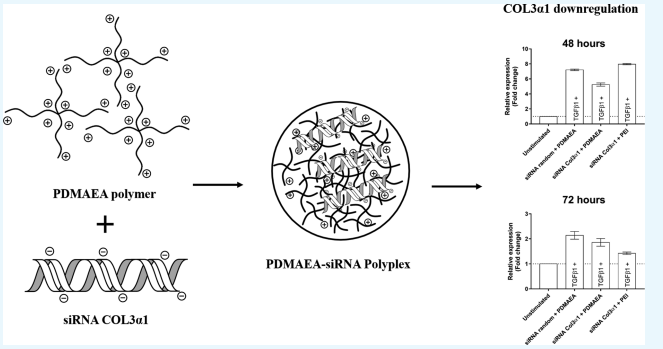
Synthesis and Formulation of Four-Arm PolyDMAEA-siRNA Polyplex for Transient Downregulation of Collagen Type III Gene Expression in TGF-β1 Stimulated Tenocyte Culture

Xin Liao, Noelia D Falcon, Ali A Mohammed, Yasmin Z. Paterson, Andrew Geoffrey Mayes, Deborah J. Guest, and Aram Saeed*

Cite This: https://dx.doi.org/10.1021/acsomega.9b03216

Read Online

ABSTRACT: The natural healing process for tendon repair is associated with high upregulation of collagen type III, leading to scar tissue and tendon adhesions with functionally deficient tendons. Gene delivery systems are widely reported as potential nanotherapeutics to treat diseases, providing a promising approach to modulate collagen type III synthesis. This work investigates a proof-of-concept four-arm cationic polymer-siRNA polyplex to mediate a transient downregulation of collagen type III expression in a tendon cell culture system. The tendon culture system was first supplemented with TGF-β1 to stimulate the upregulation of collagen type III prior to silencing experiments. The four-arm poly[2-(dimethylamino) ethyl acrylate] (PDMAEA) polymer was successfully synthesized via RAFT polymerization and then mixed with siRNA to formulate the PDMAEA-siRNA polyplexes. The formation of the polyplex was optimized for the N:P ratio (10:1) and confirmed by agarose gel electrophoresis. The size and solution behavior of the polyplex were analyzed by dynamic light scattering and zeta potential, showing a hydrodynamic diameter of 155 ± 21 nm and overall positive charge of +30 mV at physiological pH. All the polyplex concentrations used had a minimal effect on the metabolic activity of cultured cells, indicating good biocompatibility. The dose and time effects of the TGF-β1 on collagen type III gene expressions were analyzed by qPCR, showing an optimal dose of 10 ng mL^{-1} TGF-β1 and 3-fold increase of *COL3α1* expression at 48 h in cultured tenocytes. The PDMAEA-siRNA polyplex concept observed a limited yet successful and promising efficiency in silencing collagen type III at 48 h compared to PEI-siRNA. Therefore, this concept is a promising approach to reduce tissue scarring and adhesion following injuries.



INTRODUCTION

Injuries to tendon tissues are a common musculoskeletal problem that can result in severe pain, reduced mobility, and diminished quality of life in a certain patient population. Tendon injuries can arise from sudden tendon rupture or can be chronic in nature, which is widely known as tendinopathy.[1−3]

Tendon rupture can be caused by acute mechanical overloading, whereas tendinopathy occurs when the tissue is exposed to chronic overuse or in age-related tissue degeneration conditions.[4] When injuries occur, the natural healing process is complex and varies in clinical outcomes based on the anatomical location of the tendon tissue.

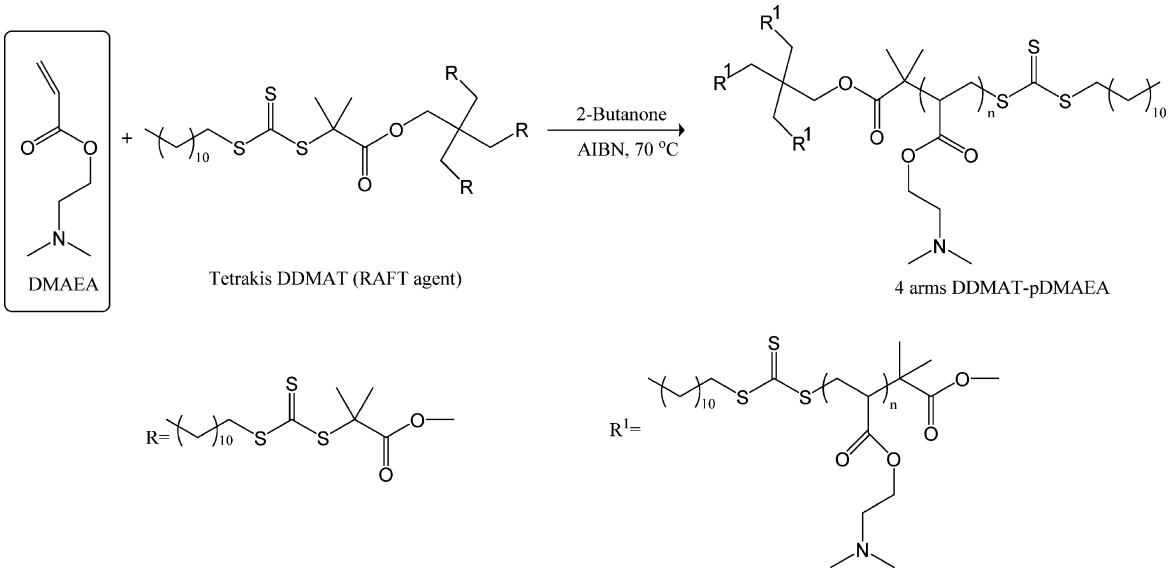
Furthermore, owing to the avascular nature of tendon tissue, the natural healing process is relatively slow and disordered resulting in a wide range of changes at both cellular and molecular levels. Typically, the wound healing process comprises three overlapping phases. These are the inflammatory phase, proliferative phase, and tissue remodeling.[5,6]

The management of cases with minor injuries could be relatively straightforward; often, a combination of moderate rest and/or medical interventions may be sufficient. In other cases, the management poses greater challenges, which may require advanced therapies. Several approaches have been investigated to augment the healing process, namely, the use of biologics (growth factors, cells therapy, siRNA) or implantable materials (scaffolds).[7,8] One of the major challenges is the limited accessibility to human tendon tissues to assess the clinical benefit of these new therapies. Consequently, studies often rely on using animal models, and equine is one of them.[9,10]

As mentioned earlier, various cellular and molecular changes occur during the natural healing process.[11,12] One of the key

Received: September 30, 2019
Accepted: January 3, 2020

Scheme 1. Schematic representation showing the chemical synthesis of a four-arm DDMAT-PDMAEA. The synthesis of a four-arm star-shaped PDMAEA polymer from DMAEA monomers using RAFT polymerization techniques. A four-arm DDMAT was used to prepare the star-shaped PDMAEA. Polymerizations were carried out in 2-butanone at 70 °C in the presence of thermal initiator AIBN



features following tendon injuries is the formation of a scar tissue (disorganized collagen fibers with mature fibroblast cells).[1−3] Williams et al. studied the histological appearance and composition of a scared tendon tissue and compared to that of a healthy tendon, using equine superficial flexor tendon.[13,14] Results showed that the cell-to-matrix ratio was increased in the scar tissue. Moreover, they reported a change in the type of collagen. In normal tendon tissue, aligned collagen type I is the most abundant protein, which constitutes 95% of the tissue. In contrast, in the reparative scar tissue, a substantial increase in the quantity of disorganized collagen type III was observed. This increase in collagen type III may lead to a weaker tensile strength when compared to that of a normal tissue. Other studies conducted in human subjects have reported similar significant changes in collagen type III concentration at the site of injury, but no significant changes in collagen type I was observed.[15−17]

Endogenous expression of growth factors is understood to orchestrate the wound healing process. Both insulin-like growth factor-I (IGF-I) and transforming growth factor-beta1 (TGF-β1) seem to play a pivotal role in the healing process, in particular on the expression of collagen types I and III.[11] Dahlgren et al. studied the temporal expression of these endogenous growth factors in equine flexor tendons following collagenase induced lesion.[18] The result of their study showed an increase in message levels for TGF-β1 in 1 week and IGF-I in 4 weeks. Similarly, message levels for both collagen types I and III were increased by week one and remained high for further 8 weeks.[18] Others have used exogenous growth factors to bolster the wound healing process by enhancing cellular expression of collagen. Nonetheless, it is plausible that in cases where the expression of collagen type III (disorganized smaller fibers) is exceeding and remains at a high undesirable level in the regenerative phase of healing process may lead to inferior mechanical properties, scar tissue formation, and/or tendon adhesion.[13,18−21] Therefore, discrete control and transient regulation of collagen type III expression during the wound healing process may be more desirable as a new therapeutic approach.

Several new approaches have been investigated to manipulate the concentration of collagen types and morphology of collagen fibers by targeting messenger RNA (mRNA). One way to control expression of collagen type III is by upregulating microRNA (miRNA) levels. Millar et al. studied miRNA29a expression in both in vitro tenocyte culture and in vivo models.[22,23] They showed that miRNA29a is able to regulate both IL-33 function and collagen type III synthesis. Furthermore, interleukin-33 (IL-33) plays an important role in the transition from type I to type III collagen synthesis in early stages of tendon remodeling. Downregulation of miRNA29a was sufficient to enhance collagen type III synthesis. In addition, small interfering RNA (siRNA) and siRNA-mediated silencing have been developed to target the gene of interest. For example, patisiran (Onpattro, Alnylam) is an FDA-approved siRNA-based treatment used in the treatment of polyneuropathy in patients with hereditary transthyretin-mediated amyloidosis, paving the way for the use of siRNA for treatment of other conditions including tendon-related disorders.[24,25]

Notwithstanding the efficacy of siRNA treatment, administration of naked siRNA is challenging as it can be degraded in the bloodstream or unable to cross cell membranes and may pose risk to induce an immunogenic response.[26,27] With that in mind, numerous siRNA delivery systems have been developed, including lipid-based and polymer-based systems.[28−30] The latter system is more accessible for customization and optimization as its components can be defined and adjusted, using a variety of starting materials and polymerization techniques.

Herein, we synthesized a new synthetic four-arm poly[2-(dimethylamino) ethyl acrylate] (PDMAEA) polymer using reversible addition-fragmentation chain transfer (RAFT).[31] The synthesized polymer was mixed with custom-designed siRNA to target collagen type III to form polyplexes (a mixture of condensed polymer and siRNA) in nanometer-sized range.

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

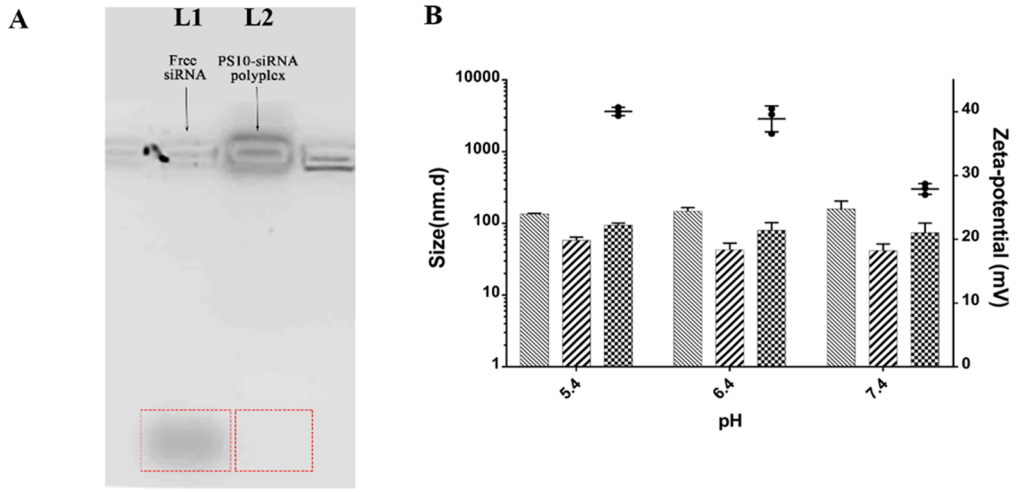


Figure 1. Confirmation of PDMAEA-siRNA polyplex formation and measurements of solution behavior. (A) Agarose gel electrophoresis showing free siRNA in the absence of the PDMAEA (lane 1) and PDMAEA-siRNA polyplexes (lane 2) at 10:1 N:P ratios. Note that agarose gel shown in (A) has been cropped, and the full-length version of the agarose gel is available in Figure S2. (B) Hydrodynamic diameter measured by dynamic light scattering (DLS). On the left *Y*-axis, the gray line, black line, and black dotted columns represent intensity, number, and volume distributions, respectively. Black dots above columns correspond to the zeta potential of the polyplexes measured at pH 5.4, 6.4, and 7.4. All data are presented as mean ± SD (n = 3). L (lane); nm.d (diameter in nanometers); mV (millivolts).

In order to investigate the toxicity profiles of these polymers and polyplex, cultured equine tenocytes were treated with various concentrations of the polyplexes. We then induced overexpression of collagen type III in the cultured cells using TGF-β1 growth factor at optimal concentration, prior to treating the cells with the polyplexes. Our result showed that the new polymer is well tolerated by the cells when compared to standard PEI, and the collagen type III message level was suppressed at 48 h of post-treatment compared to 72 h when PEI polyplex was used. This study shows a proof of concept to use a new and safe siRNA-based approach as a potential treatment by regulating the collagen type III synthesis in tendon-related disorders.

RESULTS

In this study, the aim was to use PDMAEA as the delivery vector in the format of a polyplex for the delivery of specific siRNA to knockdown the expression of *COL3α1* in TGF-β1 stimulated tenocytes as shown in Scheme S1. A four-arm cationic DMAEA polymer was successfully synthesized with a molecular weight of ∼10 kDa using our previously established protocol (Scheme 1).[31]

Characterization of PDMAEA-siRNA Polyplexes. Prior studies demonstrated the significance of particle size, surface charge, and method of preparation of polyplexes in gene transfection.[32−35] Previously, we optimized the polyplexation of PDMAEA polymers with model dsDNA.[31] The results of this study showed that a N:P ratio of 10:1 is ideal (the ratios of moles of the amine groups of cationic polymers to those of the phosphate ones of DNA) for the formation of a stable polyplex with hydrodynamic diameters of 155 ± 21 and positive zeta potential values at a relevant pH range. Hence, in this work, a N:P ratio of 10:1 for the PDMAEA-siRNA polyplexes was used. Characterization of the polyplex was carried out using agarose gel electrophoresis to confirm the polyplex formation. Figure 1A demonstrates the polyplex formation at a 10:1 N:P ratio (lane 2) compared to free siRNA in the absence of the PDMAEA polymer (lane 1). Full-length agarose gel electrophoresis is shown in Figure S2. Figure 1B demonstrates the well-defined hydrodynamic diameter size and positive zeta potential values of the PDMAEA-siRNA polyplexes measured at pH 5.4, 6.4, and 7.4, in the pH range that occur during intracellular uptake. Particle sizes are calculated from the intensity, number, and volume distributions. The number and volume distribution are marginally smaller than the intensity distribution but show a similar trend across all measured pH values.

Calculated mean sizes for the PDMAEA-siRNA polyplex were 155 ± 21, 47 ± 7, and 82 ± 17 nm for intensity, number, and volume distributions respectively. The mean zeta potential values are +41.6 ± 2.2, +38.9 ± 1.6, and +29.7 ± 1.1 for pH of 5.4, 6.4, and 7.4 respectively. These variations between intensity and number distributions are expected from DLS measurement owing to the presence of heterogeneous polyplex populations. Notably, the positive zeta potential value is greater at pH 5.4 compared to pH 7.4, likely due to the higher protonation of the amine groups on the side chains of the polymer at a lower pH value. Nonetheless, the size distributions and zeta potential values of the PDMAEA-siRNA polyplexes are within the range of reported values for polyplexes employed in transfection experiments.

Effect of the PDMAEA and PEI Polymers and PDMAEA-siRNA and PEI-siRNA Polyplexes on Cultured Tenocytes. The results in Figure 2A,B show the cytotoxicity of PDMAEA and PEI polymers as well as PDMAEA and PEI-siRNA polyplexes, as measured by the standard MTS assay, in cultured equine tenocytes, at a range of polyplex concentrations of 0.25, 2.5, 5, 10, 15, 20, 25, 30, and 50 μM and for the incubation times of 24, 48, and 72 h. All examined PDMAEA polymer and polyplex concentrations had no or minimal effects on the metabolic activity of the cultured cells compared to untreated control cells. Similarly, different incubation times of the cells with the PDMAEA polymer or polyplexes resulted in no significant changes in cellular metabolic activity. The results indicated that both PDMAEA and PDMAEA-siRNA polyplexes had low cytotoxicity and were well tolerated by the cultured equine tenocytes. In contrast, the examined PEI and PEI-siRNA polyplexes had

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

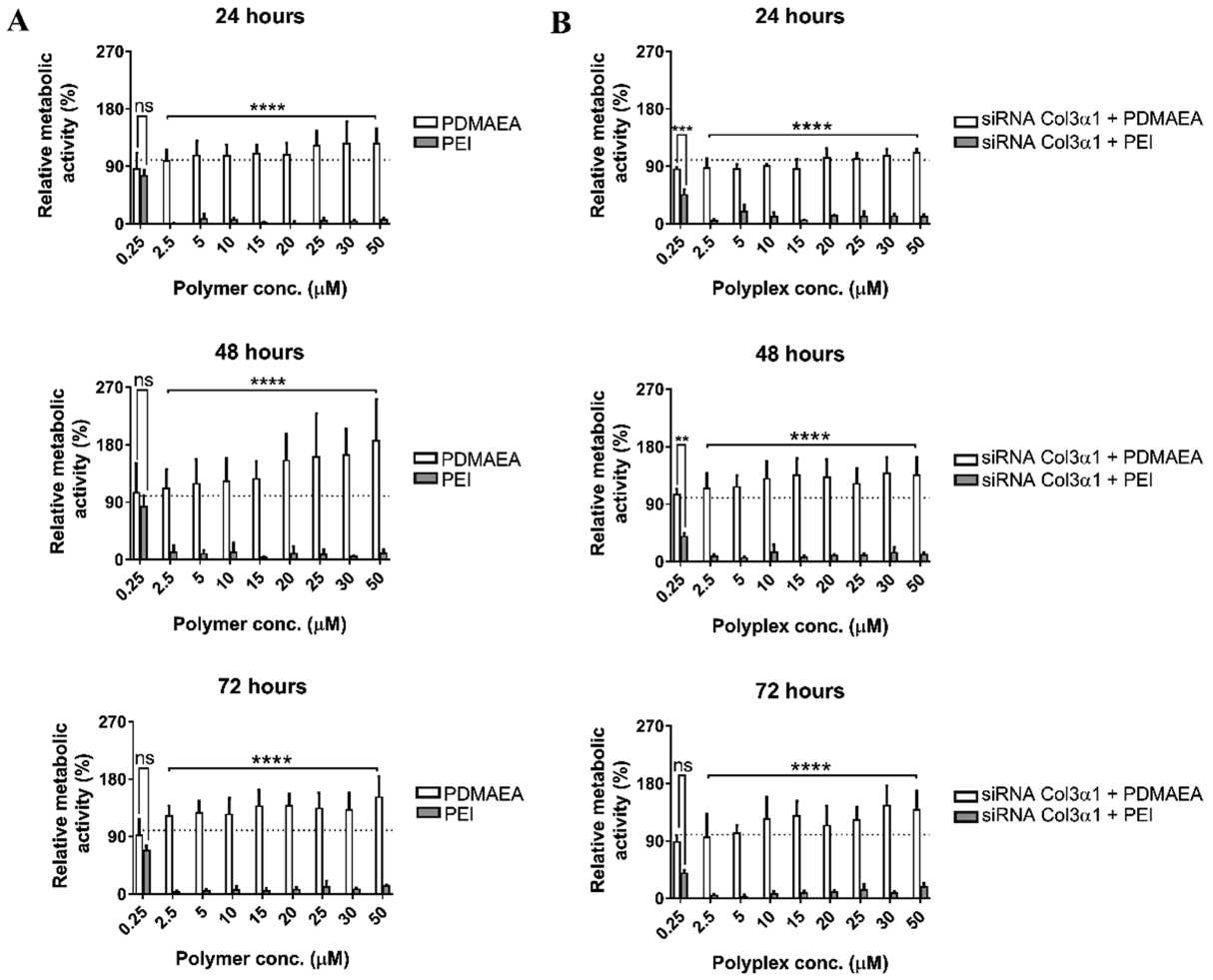


Figure 2. Effect of PDMAEA and PEI polymers and PDMAEA-siRNA and PEI-siRNA polyplexes on metabolic activity of cultured equine tenocytes. Adult equine tenocytes were treated with different concentrations of PDMAEA or PEI (A) and PDMAEA or PEI-siRNA polyplexes (B), and metabolic activity was measured by the MTS assay at 24, 48, and 72 h post-treatment. All data are presented as mean ± SD (n = 3, independent cell culture replicates), as a percentage of metabolic activity of cultured equine tenocytes normalized to untreated control cells (dashed line). Statistical analysis two-way ANOVA with Tukey's multiple comparison test was applied for displaying significant differences of the doses in between the PDMAEA and PEI polymers or PDMAEA or PEI-siRNA polyplexes. ns: not significant (p = 0.1234), **p = 0.0021, ***p = 0.0002, ****p < 0.0001. *COL3α1* (collagen 3α1); PDMAEA (polydimethylaminoethyl acrylate); siRNA (small interfering RNA); PEI (polyethyleneimine).

significant (p < 0.0001) cytotoxic effects on the cultured equine tenocytes, especially in concentrations ranging from 2.5 to 50 μM and regardless of the incubation time.

Collagen Type III Expression Levels Remain Constant with Serial Passaging of Equine Tendon Cells. The effect of serial passage on its expression in equine tendon cells was determined to ensure that collagen type III gene expression levels did not decrease over time in culture. *COL3α1* was expressed at high levels at all passages between cells isolated from different donors, although they were variable. No significant differences in *COL3α1* expression were observed at any passage between P0 and P10 (Figure 3A). In contrast, *COL1α1* has a higher expression at P0 than it does at P3 through to P10 (Figure 3B). However, *COL1α1* levels do not significantly change beyond P3 with the further passage.

TGF-β1 Treatment. TGF-β1 was used to upregulate the synthesis of collagen type III in cultured equine tenocytes. In order to optimize the dose of TGF-β1, cultured tenocytes were supplemented with different concentrations of TGF-β1 from 0.5 to 50 ng mL^{-1} for 24 h. The results in Figure 4A show the relative expression of *COL3α1* as measured by qPCR. The data showed that the relative expression of *COL3α1* (fold change) increases with increased concentration of TGF-β1 up to a 3-fold increase at 10 ng mL^{-1}. However, at higher concentrations of 25 and 50 ng mL^{-1} TGF-β1, *COL3α1* exhibits lower fold increases of 2.5- and 1.5-fold, respectively. Therefore 10 ng mL^{-1} was used in the following experiments.

In addition, to study the fold changes in *COL3α1* expression as a function of time, we examined the fold change of *COL3α1* in the cultured tenocytes, using a concentration of 10 ng mL^{-1} and incubation times of 24, 48, and 72 h. The results in Figure 4B demonstrate that *COL3α1* expression increased for all time points in samples supplemented with TGF-β1 compared to samples without TGF-β. Furthermore, the maximum *COL3α1* expression occurred after 48 h of TGF-β1 stimulation.

PDMAEA-siRNA Transfection of Equine Tenocytes. Next, the siRNA-mediated knockdown of *COL3α1* in the TGF-β1 stimulated equine tenocytes using PDMAEA-siRNA polyplex was investigated. Cells with no added TGF-β1 and PDMAEA-siRNA random polyplex were used as a negative control, whereas PEI-siRNA *COL3α1* was used as a positive control. The results in Figure 5 show the fold change in

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

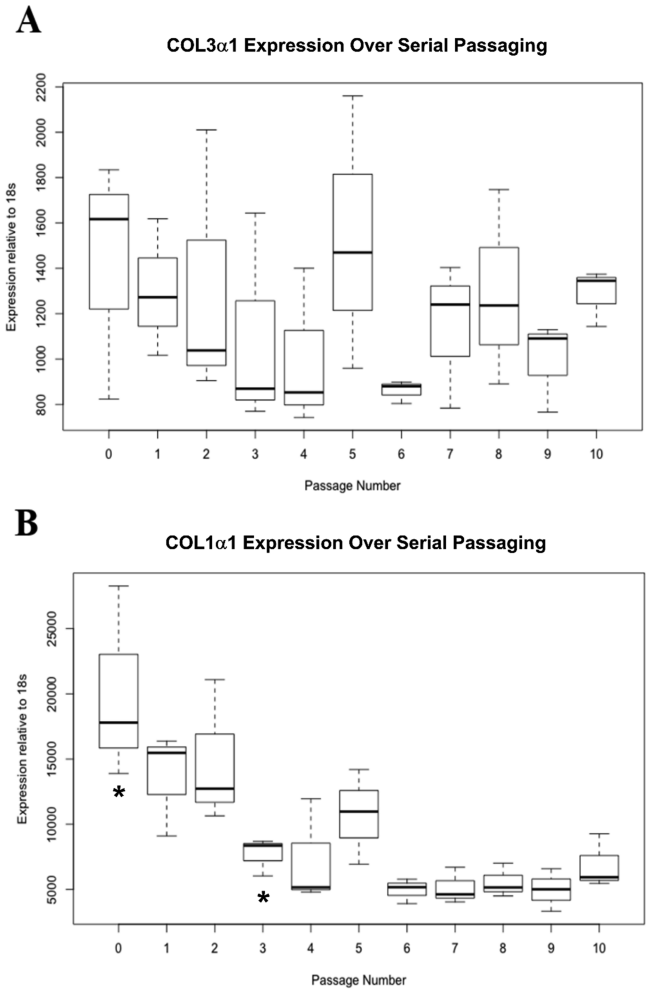


Figure 3. Collagen type III and I expression levels in serial passaging of equine tendon cells. Box and whisker plots of collagen expression over serial passaging (P0 to P10) in three independent lines of equine tendon cells. (A) *COL3α1*shows no significant differences in expression between any passages. (B) *COL1α1*expression at P0 is significantly higher than at P3 through to P10 as indicated by the asterisk. However, after P3, there is no significant change in *COL1α1* expression with the further passage. Statistical significance was tested using linear regression analysis using R (v.3.5.2).

COL3α1 as measured by PCR after 48 (Figure 5A) and 72 h (Figure 5B) compared to the non-transfected control cells cultured in the absence of TGFβ1. It can be seen that the target PDMAEA-siRNA *COL3α1* polyplex mediated a reduction in fold change of 2 in *COL3α1* expression compared to the non-target PDMAEA-siRNA-random after 48 h, providing a positive insight into the potential of the polyplex. In contrast, the target PEI-siRNA *COL3α1* polyplex showed no reduction compared to the non-target PDMAEA-siRNA random. However, at 72 h, the target PDMAEA-siRNA *COL3α1* had no silencing effect compared to non-target PDMAEA-siRNA random, but the target PEI-siRNA *COL3α1* polyplex showed a fold change of 2 reduction compared to the non-target PDMAEA-siRNA random *COL3α1*. Nonetheless, the overall fold increase of *COL3α1*in TGFβ1 treated versus untreated cells was higher at 48 h compared to 72 h.

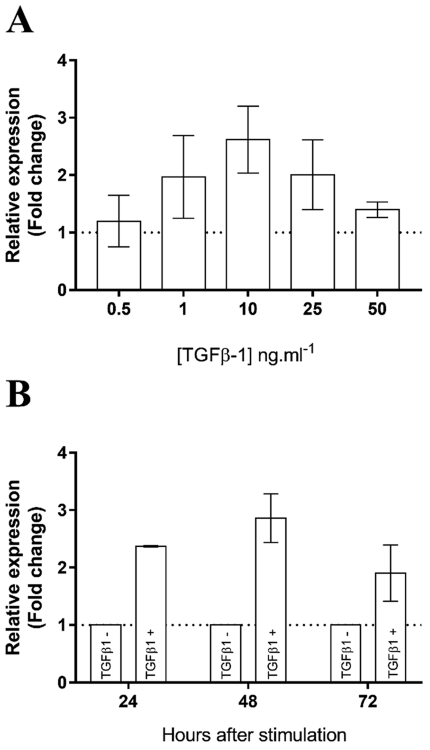


Figure 4. Dose and time effects of TGF-β1 treatment on collagen type III expression in cultured equine tenocytes. (A) Adult equine tenocytes were treated with different concentrations of TGF-β1 for 24 h, and *COL3α1* mRNA levels were analyzed by qPCR. (B) Adult equine tenocytes were treated with 10 ng mL^{-1} TGF-β1, and *COL3α1* mRNA levels were analyzed by qPCR at 24, 48, and 72 h after stimulation. Data in panels (A) and (B) are shown as relative expression (fold change) compared to untreated cells. The dashed line represents the baseline equal to 1 calculated with DCt values from untreated cells. 18S housekeeping gene expression was used to normalize the data. Data are presented as mean ± range. TGF-β1 (transforming growth factor-β1); +TGF-β1 (adult equine tenocytes treated with transforming growth factor-β1); -TGF-β1 (untreated adult equine tenocytes).

DISCUSSION

The overall aim of this study was to establish a simple formulation based on a well-defined four-arm cationic polymer for the delivery of siRNA. The intent was to target and suppress collagen type III synthesis in an ex vivo tenocyte culture. In this study, a four-arm PDMAEA cationic polymer with a molecular weight of approximately 10 kDa was chosen. This was based on a previously optimized system where we demonstrated that this specific molecular weight was able to readily form polyplexes with dsDNA molecules and reduce cytotoxicity compared to its linear counterpart or "gold-standard" transfecting agent PEI.[31] We also demonstrated that the four-arm PDMAEA polymer could form a polyplex with siRNA at a relatively low N:P ratio, likely due to the lower steric hindrance in the open four-arm structure. The hydrodynamic diameter of the formed polyplexes was investigated at a pH range that resembles the changes that occur in the cytosol. All concentrations examined of the PDMAEA polymer successfully resulted in polyplex formation with the siRNA. The resulting particles had an average hydrodynamic diameter of 155 ± 21 nm. Previous reports suggested that particle sizes less than 200 nm could be efficiently taken up by cells.[36−38] Similarly, the presence of a

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

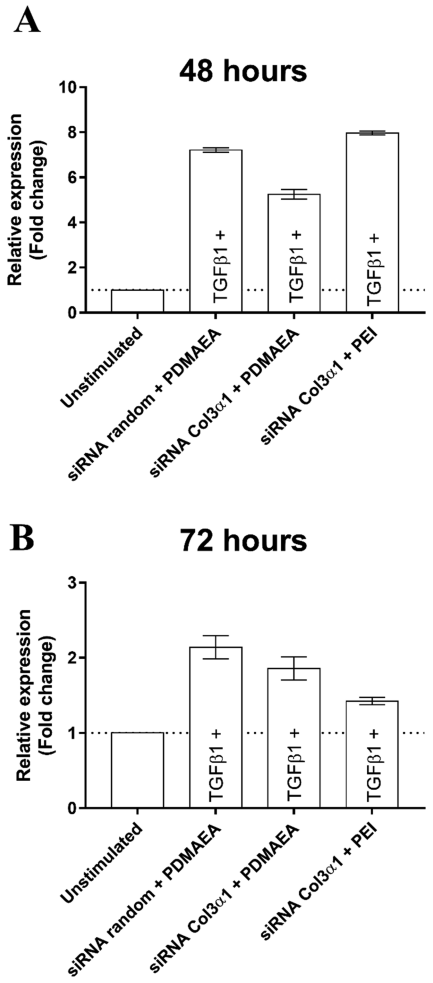


Figure 5. PDMAEA-siRNA polyplex mediated silencing of *COL3α1* in cultured equine tenocytes. Adult equine tenocytes were treated with 10 ng mL^{-1} TGF-β1 for 24 h and subsequently with PDMAEA-siRNA polyplexes in different conditions. *COL3α1* mRNA levels were analyzed by qPCR at (A) 48 and (B) 72 h post-transfection. Data in panels (A) and (B) are shown as relative expression (fold change) compared to untreated cells. The dashed line represents the baseline equal to 1, calculated with DCt values from untreated cells. 18S housekeeping gene expression was used to normalize the data. Data are presented as mean ± range. TGF-β1 (transforming growth factor-β1); +TGF-β1 (adult equine tenocytes treated with transforming growth factor-β1); -TGF-β1 (untreated adult equine tenocytes), *COL3α1* (collagen 3α1); PDMAEA (polydimethylaminoethyl acrylate); siRNA (small interfering RNA); PEI (polyethyleneimine).

positive surface charge is reported to affect cellular uptake.[39,40] The zeta potential of the polyplexes at relevant pH values above +20 mV is an indication of colloidal stability.

Previous studies showed a "tradeoff" relationship between transfection efficiency and cytotoxicity of gene delivery vectors. For example, polyethyleneimine (PEI), a non-viral vector, is reported to produce high transfection efficiencies but demonstrates significant cellular toxicity, as shown in the present work per results in Figure 2A,B. In contrast, we demonstrated that the PDMAEA polymer profile was well tolerated in cultured equine tenocytes at different concentrations and incubation times.[31] Nonetheless, the biocompatibilities of both the PDMAEA and PDMAEA-siRNA polyplexes were re-examined. There was no apparent cytotoxicity of the PDMAEA or PDMAEA-siRNA polyplexes in cultured tenocytes, rendering it a good candidate for gene targeting. The biocompatibilities of the PDMAEA and PDMAEA-siRNA polyplex were illustrated at a range of concentrations (including the concentration used in transfection). The MTS standard metabolic assay was used, which is often used to measure the cytotoxicity of molecules in cultured cells.[41] The results showed that the polymer and the polyplex concentrations used had minimal cytotoxicity toward cultured cells over prolonged incubation times up to 72 h. Therefore, the data indicate that both the PDMAEA and the PDMAEA-siRNA polyplex were well tolerated by the tenocytes and can be used as a safe proof-of-concept gene delivery vector. In contrast, the obtained results demonstrated that the metabolic activity of the cultured tenocytes was significantly hampered by all the tested concentrations of PEI polymer, except for the dose used in transfection (0.25 μM), which did not significantly reduce the metabolic activity compared to PDMAEA polymer at the same concentration. However, the effect on the metabolic activity of the PEI-siRNA polyplexes at transfection concentration (0.25 μM) was found to be significant at 24 and 48 h of incubation times, but not at 72 h, suggesting a potential recovery of the cultured tenocytes in longer periods of incubation. Similar to PEI polymer, the rest of the tested PEI-siRNA polyplex concentrations showed cytotoxic effects in the cultured tenocytes. Therefore, the results suggest that PDMAEA-siRNA polyplexes are a good candidate for gene delivery and allow for the use of a wide range of polymer concentrations, whereas PEI-siRNA polyplexes should be used at lower concentrations to avoid a greater cytotoxic effect.

Next, collagen type III silencing mediated by the polyplex was investigated. However, gene expression in tendon cells has been shown to change with culture[42,43] and can change rapidly from the expression levels found in the native tissue.[44] We, therefore, confirmed that *COL3α1* expression levels did not decline over time in culture and that the use of high passage cells in these studies would still give applicable results. We found that, while there was variation in *COL3α1* expression at each passage, it was highly expressed in all donor cell lines at all passages and that there is no significant difference in *COL3α1* expression at any passage between passage 0 and passage 10 in equine tenocytes. This is in contrast to a previous study on human tenocytes that showed that *COL3α1* expression increased with passage.[42] We also demonstrate that *COL1α1* levels drop significantly between passage 0 and passage 3 but remain constant until passage 10. Furthermore, we previously demonstrated that there is no significant difference in collagen gel contraction and response to inflammation in equine tendon cells used between passages 4 and 11.[45] Therefore, although the effect on collagen knockdown was performed in high passage cells in this current study, we believe that the results provide an accurate in vitro model.

Ideally, we would have chosen to use tenocytes isolated from rupture sites that are reported in the literature to produce more collagen type III than cells isolated from healthy tendon tissue.[46] However, in the absence of suitable donor tissue, we increased the synthesis of collagen type III using TGF-β1.[47] Since this was a new experiment, we first investigated the effect of concentration and incubation time of TGF-β1 in cultured tenocytes. Using PCR to calculate the relative expression of *COL3α1*, we were able to determine the effect of different doses of TGF-β1. From there, we determined the optimal concentration and length of application of TGF-β1 which

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

induced maximal *COL3α1* gene expression. The conditions were optimized for both PDMAEA and PEI and to allow for direct comparisons with the PCR assays. Both polymers have an equal amount and concentration of the therapeutic siRNA and were tested under the same conditions.

Next, we demonstrated that the PDMAEA-siRNA polyplex could specifically silence *COL3α1* gene expression in TGF-β1 boosted tenocyte culture conditions. As measured by PCR, the fold change in the *COL3α1* silencing effect was observed only for the polyplex formed with the target PDMAEA-siRNA *COL3α1*. However, the silencing effect was more apparent at 48 h compared to 72 h, likely since the highest induction of *COL3α1* synthesis was achieved at 48 h by TGF-β1. We also noted that PEI-siRNA polyplex mediated silencing was greatest at 72 h. The two polymers have different transfection efficacies where PEI achieves its greatest silencing at 72 h, but our polyplex achieves it at 48 h without cytotoxicity, as confirmed per the MTS assay and shown in Figure 2A,B. Although messenger RNA analysis by qPCR has been widely used as a tool to assess gene expression in tendon tissue engineering, future work could benefit from protein or other analysis to provide other quantitative methods for this study.

CONCLUSIONS

This study provides a successful proof-of-principle that the PDMAEA polymer can be used for delivery of siRNA to target collagen type III overexpression with potential application in tendon injuries. Optimizing the polyplex system can further provide a more comprehensive understanding of this technique as a specific silencing system. For example, the collagen type III knockdown was performed on tenocytes derived from one individual animal, and therefore, further biological replicates would provide greater insight into the effectiveness of this approach. Our polyplexes are well tolerated with increased concentrations suggesting that repeated doses might be possible. As tendon is avascular, systematic absorption might be less favorable in comparison to direct local injection with the aid of ultrasound scanning. Furthermore, the effect on collagen type III silencing could be studied further by determining the effect on *COL3α1* protein expression and localization. Additionally, determining the effect of applying a short pulse of TGF-β1 versus a sustained exposure and the effect of TGF-β1 over longer periods of time would provide a deeper comprehension into the potential silencing mechanism. Further work would be required to determine the impact on the ratio of collagen types III and I following *COL1α3* knockdown. Following a tendon injury, there may also be an influx of cells from outside the tendon, for example, bone-marrow-derived stromal cells. Future work should aim to investigate whether this technology can also be used to reduce *COL1α3* expression in such cell types. Studies on the dose duration and efficacy of uptake can also be examined as future progress from this work. Further studies will also investigate the development of the injectable and controlled-release system to deliver PDMAEA-siRNA polyplex over a prolonged period of time in order to control tendon adhesion and tissue scarring. This could be carried out by loading or encapsulating the polyplex into polymeric nanoparticles composed of degradable polymers, using standard double emulsion techniques, in which the rate of the polymer degradation can be used to tune the release profile.[48]

MATERIALS AND METHODS

Chemical and Biological Reagents. Four-arm star-shaped polydimethylaminoethyl acrylate (PDMAEA, *Mn*: 10000, *Mw*: 12000) was synthesized by reversible addition-fragmentation chain transfer (RAFT) as reported previously[31] and used as the transfecting agent. Collagen type III siRNA (denoted as siRNA *COL3α1*, *Mw* 13273, 10 nmol, sense strand 5′-3′ CAUCACAUAUCACUGCAAA [dT][dT], antisense strand 5′-3′ UUUGCAGUGAUAUGUGAUG [dT]-[dT]) sequences specific for equine tendon cells were purchased from Sigma-Aldrich. 18S ribosomal RNA (18Sr RNA) primer sequences were used as a housekeeping gene. A random siRNA sequence (denoted as siRNA random) to the siRNA *COL3α1* was also purchased from Sigma-Aldrich and used as a negative control. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay, Promega, U.K.), branched polyethyleneimine (denoted as PEI, 10000 Da-branched, Sigma), agarose (Sigma), Tris base (Sigma), acetic acid (Sigma), EDTA (Sigma), MTS (Promega), bovine serum albumin (BSA, Fisher), Dulbecco's modified Eagle medium (DMEM, Fisher), fetal calf serum (FCS, Fisher), trypsin (Fisher), penicillin−streptomycin solution (Fisher), L-glutamine (Fisher), HEPES (Bioreagent, 99%, Sigma), Tri Reagent solution (Invitrogen), 1-bromo-3-chloropropane (Sigma), isopropanol (Sigma), ethanol (Sigma), and gelatine solution (Sigma) were used as received. The DNA-free DNA Removal Kit (Thermo Fisher), High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher), and SYBR Green JumpStart Taq ReadyMix (Sigma) were used according to manufacturers' guidelines. Phosphate buffered saline tablets (PBS, Fisher Chemical) were used at 1× concentration in ultrapure water for cell culture purposes. Ethidium bromide was used at a concentration of 6 $\mu g\ mL^{-1}$ in 1× TAE for agarose gel electrophoresis. Paraformaldehyde (Sigma) was used at 3% concentration for immunocytochemical techniques. Adult horse tendon cells (received at passage 7) were provided by Dr. D. Guest (Animal Health Trust, U.K.) and were grown as monolayers and expanded to passage 9 for the experiments. Human recombinant TGF-β1 was purchased from Peprotech.

Preparation of PDMAEA-siRNA Polyplexes. siRNA solution was added to a microcentrifuge tube at a final concentration of 132 $\mu g\ mL^{-1}$ in HEPES buffer (100 μL, 30 mM, pH 7.4). Calculated amounts of the PDMAEA were prepared in 100 μL of HEPES buffer (30 mM, pH 7.4) and added to siRNA to form polyplex at a N:P ratio of 10:1. The polyplex solution was diluted to 1 mL with HEPES buffer resulting in a final siRNA concentration of 13.2 $\mu g\ mL^{-1}$ and a final PDMAEA concentration of 0.6 $mg\ mL^{-1}$. Solutions were mixed by vortexing for 15 s and then allowed to equilibrate for 30 min at room temperature before characterization.

Agarose Gel Electrophoresis. PDMAEA-siRNA polyplexes (8 μL of the sample with 2 μL of 1× loading dye) were loaded on a 1.5% agarose gel in Tris-acetate−EDTA (TAE, pH 8.0). Electrophoresis was performed at 80 V for 40 min in a Mini-Sub cell system (Bio-Rad Laboratories). The gel was later submerged in 0.006% ethidium bromide solution for post-cast staining for 1 hour. Bands were visualized and photographed using a ChemiDoc-It 2810 imager (U.V.P, USA). Data were collected and analyzed using the software provided.

Dynamic Light Scattering. The size and dispersity of the PDMAEA-siRNA polyplexes were determined by dynamic light scattering. 1 mL of PDMAEA-siRNA polyplex (prepared

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

in 30 mM HEPES, pH 7.4) was added to a cuvette, and the samples were examined using a Zetasizer Nano ZS (Malvern, U.K.) equipped with a He-Ne laser (4 mW, 633 nm). The measurement angle was set at 173° backscattered. The size distribution of the scattering polyplexes was determined and reported as average light intensity, volume, and number of polyplexes.

Surface Charge Measurement (Zeta Potential). The surface charge of the PDMAEA-siRNA polyplexes was measured by a Zetasizer Nano ZS (Malvern, U.K.) using a DTS 1060 folded capillary cells. The measurements were recorded in 10 mM HEPES buffer solution at pH 5.5, 6.5, and 7.4. The zeta potential measurements are reported as mean value ± standard deviation from three measurements of at least 20 runs per measurement.

Cells Metabolic Activity Assay. Adult equine tendon cells were isolated, as described previously,[49] from healthy adult horse tendons with the approval of the Animal Health Trust Ethical Review Committee (AHT_02_2012). These cells were analyzed previously for their gene and protein expression,[45,49] ability to contract a collagen gel,[50] and the effect of gene knockdown.[51] Tendon cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin−streptomycin (PS). All cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells at passage 9 were seeded at 10000 cells/0.32 cm^2 (96-well plates) and allowed to adhere for 24 h before the experiment began. The PDMAEA or PEI-siRNA polyplexes were freshly prepared as described previously at different concentrations of the PDMAEA or PEI polymer, ranging from 0.25, 2.5, 5, 10, 15, 20, 25, 30, and 50 μM. The polymer and polyplexes were incubated for 24, 48, and 72 h. To analyze the metabolic activity of the equine tenocytes, 10% MTS reagent was used according to the manufacturer's protocol. After 1 h of incubation, the absorbance was read at 490 nm in a microplate reader (Omega). For PEI containing samples, the supernatant was removed and transferred to a fresh plate before absorbance was read. All the data are presented as a percentage of cell metabolic activity compared to non-treated cells.

Collagen Type III Expression Level in Serial Passages of Equine Tendon Cells. Three independent tendon cell lines were cultured from three different horses and RNA extracted at every passage to determine the expression of *COL1α1* and *COL3α1* using qPCR.

TGF-β1 Treatment. Adult equine tendon cells (passages 7−9) were cultured in six-well plates at a density of 600000 cells/9.6 cm^2. To determine the optimal stimulation dose of TGF-β1 for collagen type III upregulation, different concentrations (0.5, 1, 10, and 25 ng mL^{-1}) were prepared in PBS (1×) containing 0.1% BSA and added to the cell culture medium. Cells were then incubated for 24 h prior to harvesting, and *COL3α1* levels were analyzed by qPCR. For transfection efficiency analysis, cells were cultured in the same conditions as described previously but using 10 ng mL^{-1} concentration of TGF-β1. Cells were then harvested after 24, 48, and 72 h, and *COL3α1* levels were analyzed by qPCR.

RNA Extraction and cDNA Synthesis. Adult equine tenocytes were directly lysed with 1 mL of Tri-reagent per well according to the manufacturer's protocol and preserved at −80 °C until analysis. After thawing, 100 μL of 1-bromo-3-chloropropane were added to each sample. After gently shaking, the tubes were incubated at room temperature for 10 min and then centrifuged at 4 °C for 20 min, in order to separate the RNA to the aqueous phase, which was then transferred to fresh tubes. 500 μL of isopropanol was added to each tube, vortexed, and incubated for 10 min at room temperature. Tubes were then centrifuged at 4 °C for 20 min, and the supernatant was discarded. RNA pellets were washed with 1 mL of 70% ethanol. After centrifugation at 4 °C, ethanol was discarded, and pellets were left to dry in air for 10 min before solubilizing the RNA in 20 μL of nuclease-free water. RNA was then treated with Ambion DNA-free DNA Removal Kit (Life Technologies, Paisley, U.K.) according to the manufacturer's instructions. RNA purity was assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher). RNA samples with values below 1.8 at a ratio of 260/280 were excluded. First-strand complementary DNA (cDNA) was made from 1 μg of RNA using High-Capacity cDNA Reverse Transcription Kit on a PTC-100 thermal cycler (Bio-Rad) using the following protocol: 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. 200 ng of synthesized cDNA in a 20 μL reaction volume was used in a quantitative real-time polymerase chain reaction (qRT PCR).

Quantitative PCR. Primer sequences used in the qPCR are reported as follows: 18S rRNA gene forward CCCAGTGAGAATGCCCTCTA, reverse TGGCTGAGCAAGGTGTTATG; *COL3α1* gene forward CTGGTGCTAATGGTGCTCCT, reverse TCTCCTTTGGCACCATTCTT. Synthesized cDNA was amplified using an SYBR Green JumpStart Taq ReadyMix master mix, and qPCRs were performed on a Rotor-Gene Q 2plex Platform (Qiagen). All qPCR reactions were performed in duplicate. PCR cycle conditions were set as follows: initial denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 15 s. At the end of the program, the temperature was reduced to 60 °C and then gradually increased by 1 °C increments up to 95 °C to produce a melt curve. Gene expression was normalized to the 18S rRNA gene expression levels using the threshold cycle (Ct) − relative quantification method ($2^{-\Delta\Delta Ct}$). The Ct values of the 18S gene did not change between the control and treated samples. Data are presented as a fold change compared to unstimulated cells. In all reactions, a standard curve including five dilutions of known cDNA was generated to ensure highly efficient product amplification. R^2 of all reactions was 0.98−0.99.

PDMAEA-siRNA Transfection of Equine Tenocytes. Adult equine tenocytes were seeded into six-well plates at a density of 600000 cells/9.6 cm^2 and allowed to adhere for 24 h. Cell culture media were then supplemented with TGF-β1 at 10 ng mL^{-1} concentration for 24 h. The PDMAEA-siRNA polyplex was prepared as described before and added to the wells to achieve a final siRNA concentration of 50 nM (this equals to 4.5 μM polyplex concentration). Cells were then harvested, RNA was extracted, and *COL3α1* and 18S mRNA levels were analyzed by qRT PCR. Control cells with no TGF-β1 and cells treated with PDMAEA-siRNA random were used as a negative control, whereas PEI-siRNA was used as a positive control. All experiments were performed in duplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03216.

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX

Experimental design for equine tenocyte culture and full length agarose gel electrophoresis (PDF)

AUTHOR INFORMATION

Corresponding Author

Aram Saeed − *University of East Anglia, Norwich, U.K.*; orcid.org/0000-0003-2903-5875; Email: Aram.saeed@uea.ac.uk

Other Authors

Xin Liao − *University of East Anglia, Norwich, U.K.*

Noelia D Falcon − *University of East Anglia, Norwich, U.K.*

Ali A Mohammed − *University of East Anglia, Norwich, U.K.*

Yasmin Z. Paterson − *Animal Health Trust, Suffolk, U.K., and University of Cambridge, Cambridgeshire, U.K.*

Andrew Geoffrey Mayes − *University of East Anglia, Norwich, U.K.*

Deborah J. Guest − *Animal Health Trust, Suffolk, U.K.*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.9b03216

Author Contributions

X.L. and N.D.F. have contributed equally and hold joint first authors. They have contributed significantly to design and execution and analysis of the experiments and writing the manuscript. Y.Z.P. contributed to Figure [3,] (collagen I vs III). A.G.M. contributed to the solution behavior of the polyplex. A.A.M. contributed to writing and revision of the manuscript. D.J.G. and A.S. have supervised the projects and contributed to manuscript writing.

Notes

The authors declare no competing financial interest.

[⊥]D.J.G. and A.S. share senior authorship.

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Tissue samples were collected with the approval of the Animal Health Trust Research Ethics Committee (AHT_02_2012), and all experiments were performed in accordance with relevant guidelines and regulations.

ACKNOWLEDGMENTS

This research was self-funded by student X.L. and his family. We also thank Miss Sheila Horseman for proofreading the manuscript draft.

REFERENCES

(1) Sharma, P.; Maffulli, N. Biology of tendon injury: healing, modeling and remodeling. *J. Musculoskeletal Neuronal Interact.* **2006**, *6*, 181−190.

(2) Sharma, P.; Maffulli, N. Tendon injury and tendinopathy: healing and repair. *J. Bone Jt. Surg.* **2005**, *87*, 187−202.

(3) Sharma, P.; Maffulli, N. Basic biology of tendon injury and healing. *Surgeon* **2005**, *3*, 309−316.

(4) Gelberman, R. H.; Steinberg, D.; Amiel, D.; Akeson, W. Fibroblast chemotaxis after tendon repair. *J. Hand Surg.* **1991**, *16*, 686−693.

(5) Massimino, M. L.; Rapizzi, E.; Cantini, M.; Libera, L. D.; Mazzoleni, F.; Arslan, P.; Carraro, U. ED2+ macrophages increase selectively myoblast proliferation in muscle cultures. *Biochem. Biophys. Res. Commun.* **1997**, *235*, 754−759.

(6) Leadbetter, W. B. Cell-matrix response in tendon injury. *Clin. Sports Med.* **1992**, *11*, 533−578.

(7) Walden, G.; Liao, X.; Donell, S.; Raxworthy, M. J.; Riley, G. P.; Saeed, A. A Clinical, Biological, and Biomaterials Perspective into Tendon Injuries and Regeneration. *Tissue Eng., Part B* **2017**, *23*, 44−58.

(8) Docheva, D.; Müller, S. A.; Majewski, M.; Evans, C. H. Biologics for tendon repair. *Adv. Drug Delivery Rev.* **2015**, *84*, 222−239.

(9) Shimomura, T.; Jia, F.; Niyibizi, C.; Woo, S. L.-Y. Antisense Oligonucleotides Reduce Synthesis of Procollagen α1 (V) Chain in Human Patellar Tendon Fibroblasts: Potential Application in Healing Ligaments and Tendons. *Connect. Tissue Res.* **2003**, *44*, 167−172.

(10) Marchant, J. K.; Hahn, R. A.; Linsenmayer, T. F.; Birk, D. E. Reduction of type V collagen using a dominant-negative strategy alters the regulation of fibrillogenesis and results in the loss of corneal-specific fibril morphology. *J. Cell Biol.* **1996**, *135*, 1415.

(11) Liu, S. H.; Yang, R. S.; al-Shaikh, R.; Lane, J. M. Collagen in tendon, ligament, and bone healing. A current review. *Clin. Orthop. Relat. Res.* **1995**, 265−278.

(12) Voleti, P. B.; Buckley, M. R.; Soslowsky, L. J. Tendon healing: repair and regeneration. *Annu. Rev. Biomed. Eng.* **2012**, *14*, 47−71.

(13) Williams, I. F.; Heaton, A.; McCullagh, K. G. Cell morphology and collagen types in equine tendon scar. *Res. Vet. Sci.* **1980**, *28*, 302−310.

(14) Williams, I. F.; McCullagh, K. G.; Silver, I. A. The distribution of types I and III collagen and fibronectin in the healing equine tendon. *Connect. Tissue Res.* **1984**, *12*, 211−227.

(15) Juneja, S. C.; Veillette, C. Defects in tendon, ligament, and enthesis in response to genetic alterations in key proteoglycans and glycoproteins: a review. *Arthritis* **2013**, *2013*, 154812.

(16) Juneja, S. C.; Schwarz, E. M.; O'Keefe, R. J.; Awad, H. A. Cellular and molecular factors in flexor tendon repair and adhesions: a histological and gene expression analysis. *Connect. Tissue Res.* **2013**, *54*, 218−226.

(17) Yang, G.; Rothrauff, B. B.; Tuan, R. S. Tendon and ligament regeneration and repair: clinical relevance and developmental paradigm. *Birth Defects Res., Part C* **2013**, *99*, 203−222.

(18) Dahlgren, L. A.; Mohammed, H. O.; Nixon, A. J. Temporal expression of growth factors and matrix molecules in healing tendon lesions. *J. Orthop. Res.* **2005**, *23*, 84−92.

(19) Platt, M. A. Tendon repair and healing. *Clin. Podiatr. Med. Surg.* **2005**, *22*, 553−560.

(20) Dowling, B. A.; Dart, A. J.; Hodgson, D. R.; Smith, R. K. W. Superficial digital flexor tendonitis in the horse. *Equine Vet. J.* **2000**, *32*, 369−378.

(21) Crevier-Denoix, N.; Collobert, C.; Pourcelot, P.; Denoix, J. M.; Sanaa, M.; Geiger, D.; Bernard, N.; Ribot, X.; Bortolussi, C.; Bousseau, B. Mechanical properties of pathological equine superficial digital flexor tendons. *Equine Vet. J.* **1997**, *29*, 23−26.

(22) Millar, N. L.; Watts, A. E.; Akbar, M.; Hughes, T.; Kitson, S.; Gilchrist, D. S. MicroRNA-29a in Equine Tendinopathy − A Translational Target. *Equine Vet. J.* **2016**, *48*, 27.

(23) Watts, A. E.; Millar, N. L.; Platt, J.; Kitson, S. M.; Akbar, M.; Rech, R.; Griffin, J.; Pool, R.; Hughes, T.; McInnes, I. B.; Gilchrist, D. S. MicroRNA29a Treatment Improves Early Tendon Injury. *Mol. Ther.* **2017**, *25*, 2415−2426.

(24) Hoy, S. M. Patisiran: First Global Approval. *Drugs* **2018**, *78*, 1625−1631.

(25) Suhr, O. B.; Coelho, T.; Buades, J.; Pouget, J.; Conceicao, I.; Berk, J.; Schmidt, H.; Waddington-Cruz, M.; Campistol, J. M.; Bettencourt, B. R.; Vaishnaw, A.; Gollob, J.; Adams, D. Efficacy and safety of patisiran for familial amyloidotic polyneuropathy: a phase II multi-dose study. *Orphanet J. Rare Dis.* **2015**, *10*, 109.

(26) DeVincenzo, J.; Lambkin-Williams, R.; Wilkinson, T.; Cehelsky, J.; Nochur, S.; Walsh, E.; Meyers, R.; Gollob, J.; Vaishnaw, A. A randomized, double-blind, placebo-controlled study of an RNAi-based

therapy directed against respiratory syncytial virus. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 8800−8805.
(27) Kanasty, R. L.; Whitehead, K. A.; Vegas, A. J.; Anderson, D. G. Action and reaction: the biological response to siRNA and its delivery vehicles. *Mol. Ther* **2012**, *20*, 513−524.
(28) Schroeder, A.; Levins, C. G.; Cortez, C.; Langer, R.; Anderson, D. G. Lipid-based nanotherapeutics for siRNA delivery. *J. Intern. Med.* **2010**, *267*, 9−21.
(29) Tucker, B. S.; Sumerlin, B. S. Poly(N-(2-hydroxypropyl) methacrylamide)-based nanotherapeutics. *Polym. Chem.* **2014**, *5*, 1566−1572.
(30) Knežević, N. Ž.; Durand, J.-O. Targeted Treatment of Cancer with Nanotherapeutics Based on Mesoporous Silica Nanoparticles. *ChemPlusChem* **2015**, *80*, 26−36.
(31) Liao, X.; Walden, G.; Falcon, N. D.; Donell, S.; Raxworthy, M. J.; Wormstone, M.; Riley, G. P.; Saeed, A. A direct comparison of linear and star-shaped poly(dimethylaminoethyl acrylate) polymers for polyplexation with DNA and cytotoxicity in cultured cell lines. *Eur. Polym. J.* **2017**, *87*, 458−467.
(32) Saeed, A. O.; Magnusson, J. P.; Moradi, E.; Soliman, M.; Wang, W.; Stolnik, S.; Thurecht, K. J.; Howdle, S. M.; Alexander, C. Modular Construction of Multifunctional Bioresponsive Cell-Targeted Nanoparticles for Gene Delivery. *Bioconjugate Chem.* **2011**, *22*, 156−168.
(33) Soliman, M.; Nasanit, R.; Abulateefeh, S. R.; Allen, S.; Davies, M. C.; Briggs, S. S.; Seymour, L. W.; Preece, J. A.; Grabowska, A. M.; Watson, S. A.; Alexander, C. Multicomponent Synthetic Polymers with Viral-Mimetic Chemistry for Nucleic Acid Delivery. *Mol. Pharmaceutics* **2012**, *9*, 1−13.
(34) Aied, A.; Zheng, Y.; Newland, B.; Wang, W. Beyond Branching: Multiknot Structured Polymer for Gene Delivery. *Biomacromolecules* **2014**, *15*, 4520−4527.
(35) Zhao, T.; Zhang, H.; Newland, B.; Aied, A.; Zhou, D.; Wang, W. Significance of Branching for Transfection: Synthesis of Highly Branched Degradable Functional Poly(dimethylaminoethyl methacrylate) by Vinyl Oligomer Combination. *Angew. Chem., Int. Ed.* **2014**, *53*, 6095−6100.
(36) Oh, N.; Park, J.-H. Endocytosis and exocytosis of nanoparticles in mammalian cells. *Int. J. Nanomed.* **2014**, *9*, 51−63.
(37) Shang, L.; Nienhaus, K.; Nienhaus, G. U. Engineered nanoparticles interacting with cells: size matters. *J. Nanobiotechnol.* **2014**, *12*, 5−5.
(38) Oyewumi, M. O.; Kumar, A.; Cui, Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. *Expert Rev. Vaccines* **2010**, *9*, 1095−1107.
(39) Fröhlich, E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int. J. Nanomed.* **2012**, *7*, 5577−5591.
(40) Yue, Z.-G.; Wei, W.; Lv, P.-P.; Yue, H.; Wang, L.-Y.; Su, Z.-G.; Ma, G.-H. Surface Charge Affects Cellular Uptake and Intracellular Trafficking of Chitosan-Based Nanoparticles. *Biomacromolecules* **2011**, *12*, 2440−2446.
(41) Wang, W.; Naolou, T.; Ma, N.; Deng, Z.; Xu, X.; Mansfeld, U.; Wischke, C.; Gossen, M.; Neffe, A. T.; Lendlein, A. Polydepsipeptide Block-Stabilized Polyplexes for Efficient Transfection of Primary Human Cells. *Biomacromolecules* **2017**, *18*, 3819−3833.
(42) Yao, L.; Bestwick, C. S.; Bestwick, L. A.; Maffulli, N.; Aspden, R. M. Phenotypic drift in human tenocyte culture. *Tissue Eng.* **2006**, *12*, 1843−1849.
(43) Shukunami, C.; Takimoto, A.; Nishizaki, Y.; Yoshimoto, Y.; Tanaka, S.; Miura, S.; Watanabe, H.; Sakuma, T.; Yamamoto, T.; Kondoh, G.; Hiraki, Y. Scleraxis is a transcriptional activator that regulates the expression of Tenomodulin, a marker of mature tenocytes and ligamentocytes. *Sci. Rep.* **2018**, *8*, 3155.
(44) Kuemmerle, J. M.; Theiss, F.; Okoniewski, M. J.; Weber, F. A.; Hemmi, S.; Mirsaidi, A.; Richards, P. J.; Cinelli, P. Identification of Novel Equine (Equus caballus) Tendon Markers Using RNA Sequencing. *Genes* **2016**, *7*, 97.
(45) McClellan, A.; Evans, R.; Sze, C.; Kan, S.; Paterson, Y.; Guest, D. A novel mechanism for the protection of embryonic stem cell derived tenocytes from inflammatory cytokine interleukin 1 beta. *Sci Rep.* **2019**, *9*, 2755.
(46) Maffulli, N.; Moller, H. D.; Evans, C. H. Tendon healing: can it be optimised? *Br. J. Sports Med.* **2002**, *36*, 315.
(47) Stallmach, A.; Schuppan, D.; Riese, H. H.; Matthes, H.; Riecken, E. O. Increased collagen type III synthesis by fibroblasts isolated from strictures of patients with Crohn's disease. *Gastroenterology* **1992**, *102*, 1920−1929.
(48) Saeed, A. O.; Dey, S.; Howdle, S. M.; Thurecht, K. J.; Alexander, C. One-pot controlled synthesis of biodegradable and biocompatible co-polymer micelles. *J. Mater. Chem.* **2009**, *19*, 4529−4535.
(49) Barsby, T.; Guest, D. Transforming growth factor beta3 promotes tendon differentiation of equine embryo-derived stem cells. *Tissue Eng., Part A* **2013**, *19*, 2156−2165.
(50) Barsby, T.; Bavin, E. P.; Guest, D. J. Three-dimensional culture and transforming growth factor beta3 synergistically promote tenogenic differentiation of equine embryo-derived stem cells. *Tissue Eng., Part A* **2014**, *20*, 2604−2613.
(51) Bavin, E. P.; Atkinson, F.; Barsby, T.; Guest, D. J. Scleraxis Is Essential for Tendon Differentiation by Equine Embryonic Stem Cells and in Equine Fetal Tenocytes. *Stem Cells Dev.* **2017**, *26*, 441−450.

https://dx.doi.org/10.1021/acsomega.9b03216
ACS Omega XXXX, XXX, XXX−XXX