Injectable biomimetic liquid crystalline scaffolds enhance muscle stem cell transplantation


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Muscle stem cells are a potent cell population dedicated to efficacious skeletal muscle regeneration, but their therapeutic utility is currently limited by mode of delivery. We developed a cell delivery strategy based on a supramolecular liquid crystal formed by peptide amphiphiles (PAs) that encapsulates cells and growth factors within a muscle-like unidirectionally ordered environment of nanofibers. The stiffness of the PA scaffolds, dependent on amino acid sequence, was found to determine the macroscopic degree of cell alignment templated by the nanofibers in vitro. Furthermore, these PA scaffolds support myogenic progenitor cell survival and proliferation and they can be optimized to induce cell differentiation and maturation. We engineered an in vivo delivery system to assemble scaffolds by injection of a PA solution that enabled coagulation of scaffold nanofibers with endogenous myofibers. These scaffolds locally retained growth factors, displayed degradation rates matching the time course of muscle tissue regeneration, and markedly enhanced the engraftment of muscle stem cells in injured and noninjured muscles in mice.

Significance

Most research aiming to achieve muscle regeneration focuses on the biology of “muscle stem cells,” but delivery methods that enhance transplantation efficiency of these cells are at early stages. We report on a liquid crystalline scaffold that encapsulates the cells and gels upon injection in vivo without requiring an external stimulus. As a unique structural feature, the scaffold contains nanofibers that align preferentially with surrounding natural muscle fibers. The biomimetic scaffold can have a stiffness that matches that of muscle, has great ability to retain growth factors, and has a biodegradation rate that is compatible with regeneration time scales. Most importantly, the scaffold enhances engraftment efficiency of the cells in injured muscle, and without injury when combined with growth factors.

Muscle stem cells are a potent cell population dedicated to efficacious skeletal muscle regeneration, but their therapeutic utility is currently limited by mode of delivery. We developed a cell delivery strategy based on a supramolecular liquid crystal formed by peptide amphiphiles (PAs) that encapsulates cells and growth factors within a muscle-like unidirectionally ordered environment of nanofibers. The stiffness of the PA scaffolds, dependent on amino acid sequence, was found to determine the macroscopic degree of cell alignment templated by the nanofibers in vitro. Furthermore, these PA scaffolds support myogenic progenitor cell survival and proliferation and they can be optimized to induce cell differentiation and maturation. We engineered an in vivo delivery system to assemble scaffolds by injection of a PA solution that enabled coagulation of scaffold nanofibers with endogenous myofibers. These scaffolds locally retained growth factors, displayed degradation rates matching the time course of muscle tissue regeneration, and markedly enhanced the engraftment of muscle stem cells in injured and noninjured muscles in mice.
amphiphile (PA) nanofibers that can be mixed with cells and sheared in electrolytic media to form gels with nanofiber alignment (25). Here we identify some of the physical properties required for the formation of PA-based liquid crystalline solutions and describe the supramolecular crafting of an injectable biomimetic scaffold of PA nanofibers to encapsulate MuSCs as a combined matrix-cell therapy. We sought to design an injectable PA scaffold with the microstructural organization and stiffness of myofibers in muscle (21, 22, 26). We hypothesized that these dual biomimetic features would enhance MuSC transplantation and therapy.

Results

Liquid Crystalline Scaffolds Arise from Solutions with Specific PA Nanostructure Size and Zeta Potential. PAs consisting of an aliphatic palmitoyl tail covalently attached to a peptide sequence self-assemble in solution at neutral pH, typically yielding a suspension of high aspect-ratio nanofibers (27) (Fig. 1A and SI Appendix, Fig. S1A). Upon annealing, certain PA nanofibers elongate effectively to “infinite” length as they reach their thermodynamically stable state, according to PA concentration and ionic strength (28). We have confirmed this for three previously described PAs by imaging them by cryogenic transmission electron microscopy (CryoTEM) before and after annealing. After annealing, it was very hard to find nanofiber ends, especially in the same fiber, making fiber length measurements impossible (SI Appendix, Figs. S1A and B). We have previously observed that lyotropic liquid crystals made of annealed PA (aPA) solutions contained very long PA nanofibers (25). However, not all aPA solutions displayed liquid crystalline behavior (25). We have also previously observed that by changing the amino acid sequence in the β-sheet-forming region of PA molecules, we could modify the stiffness of the resulting nonannealed PA gels (29). Since we wanted to create liquid crystalline scaffolds varying in stiffness, we first investigated the nanoscale structure of several negatively charged aPAs (Fig. 1B and SI Appendix, Figs. S2–S6). We found that not all aPAs formed infinite-length nanofibers when dissolved at 13 mM in a salt-containing solution. In fact, C16A3V3E3 formed “short” nanofibers and C16V4A2E3 formed even shorter supramolecular structures (Fig. 1B). The PAs that did form long nanofibers, in this case twisted ribbons, were C16V2A4E3, C16V3A3E3, and C16V2A2E2 (Fig. 1B). Coincidentally, the 13-mM solutions of these three aPAs were also found to display domains of birefringence, a blueprint of their liquid crystalline state (Fig. 1C). Moreover, these three aPA solutions, but not those formed by C16A3V3E3 or C16V4A2E3, could be gelled

![Fig. 1. Chemical and physical properties of peptide amphiphile solutions. (A) Chemical structure of a peptide amphiphile (PA) used to create scaffolds and molecular graphics representation of PA molecules - assembled into a supramolecular nanofiber. (B) Cryo transmission electron micrographs of different annealed 13-mM PA (aPA) solutions. (Scale bar, 100 nm.) (C) Birefringence images of different 13-mM aPA solutions. (Scale bars, 1,500 μm and 500 μm for the Top and Bottom images, respectively.) Average size (D) and zeta potential (E) measurements of annealed or non-annealed 0.13-mM PA solutions. The individual replicate measurements together with the mean (horizontal bar) are shown. *P < 0.05; **P < 0.01; ****P < 0.0001; ns, nonsignificant; two-way ANOVA with Bonferroni post hoc test.]
into self-sustaining “noodle”-shaped scaffolds. These gel scaffolds contained oriented or nonoriented nanofibers upon extrusion into or contact with another solution containing divalent ions, respectively, as previously described (25) (SI Appendix, Fig. S7A). These observations suggested that PA nanofiber length was a contributing factor to the liquid crystalline state of the solution. In fact nonannealed PA solutions, which are expected to contain shorter nanofibers (SI Appendix, Fig. S1), lacked birefringence except for C13V3A3E3 (SI Appendix, Fig. S7B).

To characterize further changes in the nanoscale structure of fibers upon annealing, we performed dynamic light scattering (DLS) and zeta potential measurements on 0.13-mM solutions (Fig. 1 D and E). Since we found the nanostructures to be the same as their 13-mM counterparts (SI Appendix, Fig. S7C), we believe these measurements are indicative of nanostructure behavior at both concentrations. When fit to a protein-size model, the average size of the nanostructures, as measured by DLS, significantly increased for all PAAs after annealing except for C16V4A2E3, which decreased (Fig. 1D). In the case of C13A3V3E3, even though the size increase was significant, it was also significantly lower than the other three aPAs (C16V2A4E3, C13V3A3E3, and C16V2A2E2) (Fig. 1D). We then measured the zeta potential of these 0.13-mM PA solutions and found that nonannealed C13V3A3E3 was significantly less negative than both C16V2A4E3 and C16V2A2E2 (Fig. 1E). The fact that nonannealed C13V3A3E3 displayed birefringence (SI Appendix, Fig. S7D), but its DLS size was not different from C16V2A2E2 (Fig. 1D), suggests that PA solutions containing short, negatively charged PA nanofibers might also display liquid crystalline behavior if their zeta potential is not “too” negative. Together, these results suggest that DLS and zeta potential measurements can be used to predict which PA solutions, annealed or not, will display liquid crystalline behavior. However, elucidating the exact contributions of each factor will require future studies and it is beyond the scope of this work.

Survival of Myogenic Progenitors Encapsulated in PA Scaffolds with Different Degrees of Stiffness. As described above, isotropic liquid crystals formed by PA nanostructures and gelled by extruding a PA solution into another solution with divalent ions organize into a hierarchical structure of oriented nanofibers that partially emulates the architecture of macroscopically oriented muscle tissue (SI Appendix, Fig. S7A). We mixed C2C12-GFP mouse skeletal muscle progenitor cells (myoblasts) with aPA solutions and gelled them concomitantly to the application of a shear force by extrusion of the mixture through a pipette tip. We observed that these constructs contained oriented nanofibers, parallel to the direction of the applied shear force, and cells were uniformly distributed within and between nanofibers (Fig. 2A). These aPA/cell constructs also conserved most of their birefringence compared with their nonseeded counterparts, attesting to the maintenance of long-range oriented domains in the presence of cells (SI Appendix, Fig. S8). We then measured the stiffness of “bulk” gels generated by 13-mM aPA solutions and observed shear storage moduli (G′) spanning a range of two orders of magnitude, from 104 to 106 Pa (Fig. 2B and SI Appendix, Fig. S9A). Since the extruded constructs contain nanofibers oriented mostly in the same direction, cells embedded in those gels might experience anisotropic mechanics. Measurements to control for orientational artifacts on stiffness (21, 22, 26), we tested in vitro at different time points if gel stiffness had an effect on myogenic cell survival within oriented aPA/cell constructs. We examined low (~3 kPa), mid (~9 kPa), and high (~15 kPa) G′ aPAs and found that viability in all conditions was >85%, and both the mid G′ and the high G′ aPA scaffolds supported slightly higher cell viability than the low G′ (Fig. 2 D and E).

aPA Gel Nanofiber Alignment and Stiffness Direct the Alignment, Proliferation, and Maturation of Myogenic Progenitors. A number of natural (30) or synthetic (31, 32) biomaterials engendered with oriented fibrous structures can promote elongation, fusion, and differentiation of myoblasts that are seeded on top of them. For myoblasts embedded in randomly oriented natural (33) and synthetic (34) biomaterials, the application of passive force (or stretch) is necessary to promote the same effects. We hypothesized that the anisotropic nanofiber orientation within the aPA scaffolds, combined with their biomimetic stiffness (26), would serve as topological and mechanical cues to promote encapsulated myoblast alignment and differentiation, without additional applied forces.

To determine if aPA stiffness directed myoblast alignment, we fabricated aPA/cell constructs containing C2C12-GFP cells at 20,000 cells per microliter in oriented aPA scaffolds with low, mid, and high G′ or in nonoriented mid G′ aPA scaffolds. We then imaged the constructs after 2, 4, and 7 d in culture in low-serum differentiation media (DM) (Fig. 3A). Using a freely available plugin in Fiji we extracted a measure of alignment based on directionality analysis (Materials and Methods and Fig. 3B) that we termed “order parameter” (Fig. 3C). Compared with the nonoriented scaffold, the three oriented aPA scaffolds induced a higher degree of cell alignment and the mid and high G′ aPA scaffolds induced even greater alignment compared with the low G′ one at all days of culture (Fig. 3A and C). This was more clearly visualized with confocal fluorescence stacks and scanning electron micrographs of C2C12-GFP cells in either oriented or nonoriented mid G′ aPA gels (SI Appendix, Figs. S10 and Movie S1). As cell alignment decreased by day 7 (Fig. 3C), we hypothesized that cell activity and growth led to increased aPA scaffold remodeling and decreased cell accessibility to the scaffold’s oriented nanofibers. To test this hypothesis, we fabricated aligned mid G′ aPA/cell constructs containing different densities of C2C12-GFP cells and imaged them at days 1 and 4 (SI Appendix, Fig. S11A). As expected, the degree of cell alignment was inversely proportional to cell density at either day in culture, supporting our nanofiber accessibility hypothesis (SI Appendix, Fig. S11 B–D). We also observed that when low and mid G′ aPA/cell constructs (50,000 cells per microliter) were kept in growth media (GM) instead of DM, they were more extensively remodeled by the cells, and that some cells were able to escape from the aPA scaffold and attach to the underlying tissue culture plate (SI Appendix, Fig. S12), mirroring the behavior of muscle stem cells as they migrate away from their associated myofibers after isolation and culture (35). Given these observations, we used an intermediate loading density (20,000 cells per microliter) to optimally balance scaffold stability and total cell numbers for subsequent C2C12 differentiation experiments.

To determine if cell alignment and differentiation were correlated, we maintained the aPA/cell constructs in DM and stained them at day 10 in culture for myosin heavy chain (MHC) and sarcomeric alpha-actinin (ACTN) to identify mature myogenic cells. We observed MHC and ACTN expression in elongated cells often spanning several cell nuclei in the mid G′ and high G′ (ACTN not tested) oriented aPA scaffolds, suggesting that cell fusion, typical of myotube maturation, coincided with cell differentiation (Fig. 3D).
In nonoriented mid G’ and oriented low G’ aPA scaffolds, some cells stained positive for MHC, but they did not appear elongated or fused with each other, suggesting that both nanofiber orientation and gel stiffness are needed for these outcomes (Fig. 3D).

To assess C2C12-GFP proliferation in aPA scaffolds, we maintained aPA/cell constructs for 8 d in culture in DM and then counted the number of proliferating cells as measured by 24 h of EdU incorporation. We observed EdU+ cells in all three oriented aPA/cell constructs but the frequency was highest in nonoriented mid G’ scaffolds (Fig. 3E). Interestingly, we found cell proliferation to be significantly reduced in nonoriented mid G’ scaffolds (Fig. 3D). Overall, the oriented mid G’ and high G’ aPA scaffold demonstrated optimal structural and mechanical features for promoting myogenic cell proliferation and differentiation, but we selected the mid G’ aPA to further test its potency as a biomimetic scaffold for muscle cell transplantation therapy.

**Development of an Injection Apparatus to Form Aligned Biomimetic Scaffolds in Vivo.** PA solutions can be gelled in vivo by divalent ions within tissues. To maintain aPA nanofiber orientation parallel to the injection track when the needle’s inner diameter matched the syringe’s inner diameter (Materials and Methods and SI Appendix, Fig. S13). To monitor the localization of biomimetic scaffolds in vivo, we injected scaffolds mixed with Evans blue dye (EBD) or scaffolds containing 5 mol% of a PA molecule with a covalently attached gadolinium [Gd(III)] label (36, 37) (Fig. 4H) into the tibialis anterior (TA) muscles of C57BL/6 mice. The paramagnetic Gd(III) chelate produces positive contrast in magnetic resonance imaging (MRI), a technique providing unparalleled spatial resolution in vivo (38). After harvesting, we examined the tissues by macroscopic inspection or MRI of EBD-labeled or Gd(III)-labeled scaffolds. We found that biomimetic scaffolds were generated throughout the length of the recipient TA muscle and parallel to its long axis (Fig. 4 C, D, and I). To further assess the biomimetic scaffold’s nanofiber orientation in vivo, we injected 1 μL of aPA solution into TA muscles and processed them for TEM. We observed that large domains within biomimetic scaffolds contained elongated nanofibers in parallel to the longitudinal direction of the neighboring endogenous tissue myofibers, thus confirming the generation of nanofiber orientation in vivo (Fig. 4 E–G).

**Degradation Properties of Biomimetic Scaffolds in Vivo.** Previous histological studies from our laboratory suggested that PA gels can remain in recipient tissues for up to a month (39, 40). Here, to track
and quantify the biomimetic scaffold’s degradation in vivo over time, we made use of scaffolds containing the Gd(III) label (36). In addition to MRI, the amount of Gd(III) label can also be quantified with great sensitivity using inductively coupled plasma mass spectrometry (ICP-MS) because of the negligible endogenous concentration of Gd(III) (41). Hence, we injected TA muscles of mice with Gd(III)-labeled scaffolds (Fig. 4H) and imaged them by MRI at 1, 4, 8, and 15 d (Fig. 4H). The Gd(III)-labeled scaffold was identifiable during the initial 8 d, but its signal approached background levels by day 15. Similar results were obtained after acquiring T1 relaxation maps (SI Appendix, Fig. S14) and plotting the averaged T1 values (Fig. 4J). To perform a more precise measurement of scaffold clearance from the tissue, we injected Gd(III)-labeled or nonlabeled scaffolds containing the same molar amount of unbound Gd(III) chelate (Prohance) and measured their Gd(III) content by ICP-MS at several time points up to 24 d (Fig. 4K). We

Fig. 3. aPA scaffold nanofiber alignment and stiffness modulate the alignment, maturation, and proliferation of C2C12-GFP cells. C2C12-GFPs were encapsulated at a density of 20,000 cells per microliter in nonaligned mid G′ aPA scaffolds or in aligned, aPA scaffolds with different stiffness magnitudes (low, mid, or high G′) and cultured for several days. (A) Representative confocal fluorescent micrographs of calcein AM-stained C2C12 cells in aPA scaffolds at different time points showing differential elongation of cells or cell clusters. (Scale bar, 200 μm.) (B) Directionality analysis from the images in A. The histograms show the angular distribution of the directions in which cells or cell clusters elongate (black bars) and the Gaussian equation that fits the data (red line). (C) As a measure of directionality, we divided the maximum by the minimum value of the Gaussian fit to calculate an order parameter (mean ± SEM). When this order parameter is 1 (red dotted line), there is no preferred direction in the sample; greater values indicate greater directionality. *P < 0.5; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, nonsignificant; one-way ANOVA with Bonferroni post hoc test. (D) Representative confocal fluorescent micrographs of C2C12-GFP cells after 10 d in culture in aPA scaffolds displaying GFP (green) and stained for total nuclei (propidium iodide, red) and myosin heavy chain (MF20 antibody) or alpha-actinin (ACTN) (blue). White arrows point to differentiated cells or cluster of cells that have likely fused with each other. (Scale bar, 200 μm.) (E, Left) Representative confocal micrographs of C2C12-GFP cells at day 8 in culture in aPA scaffolds displaying GFP (green) and stained for EdU incorporation (red) and total nuclei (DAPI, blue). (Scale bar, 250 μm.) (Right) Quantification of the percentage of cells that have incorporated EdU (24-h incubation), from the images above; ***P < 0.001; ns, nonsignificant; one-way ANOVA with Bonferroni post hoc test.
found ∼85% of Gd(III) in the tissue 2 h after injection when the Gd(III)-labeled scaffold was used and decreased steadily over time to ∼30% by day 24. After fitting a one-phase decay equation to the data, we found that the Gd(III)-labeled scaffold’s half-life in the TA muscle was 13.5 d, indicating that the scaffold is degrading during the time window of matrix remodeling typical of muscle regeneration (42). In contrast, we observed that the unbound chelate (Prohance) in the nonlabeled scaffolds was quickly cleared since Gd(III) was essentially absent by 2 h after injection (Fig. 4K), proving that ICP-MS measured the clearance of scaffold-bound Gd(III) and not free Gd(III) ion/chelate. It is interesting to note that while the Gd (III)-labeled scaffold was no longer identifiable at day 15 by MRI, the actual drop in Gd(III) content as measured by ICP-MS from day 8 to day 15 was only ∼10%. This suggests that either a portion of the Gd(III)-labeled scaffold diffused away from the injection site but remained in the muscle tissue for days before completely dissipating, or that there is still ∼40% of the scaffold in the injection site that cannot be observed by MRI due to a sensitivity limit in this configuration.

Encapsulation of Growth Factors by Biomimetic Scaffolds Enhances Primary Myoblast Proliferation in Vitro. We assessed the effects of the biomimetic scaffold on primary mouse myoblast encapsulation and transplantation. Primary myoblasts were ∼100% viable in biomimetic scaffolds when cultured in GM for 5 d but their viability decreased to ∼90% after switching to DM (SI Appendix, Fig. S15), suggesting that their survival is sensitive to growth factors (GFs) in the media. Moreover, we found that myoblasts in GM were highly proliferative at day 1 (∼90%) but that proliferation decreased over
time, reaching <60% at day 5 (Fig. 5A and B). This decrease appeared to be correlated with increased differentiation, as evidenced by MHC immunostaining (Fig. 5A), resembling the in vivo myogenic regenerative progression (1). We hypothesized that the encapsulation of GFs within biomimetic scaffolds could enhance primary myoblast proliferation and engraftment in environments with low serum GFs. We first tested this possibility in vitro by culturing myoblasts in biomimetic scaffolds using DM alone, DM with FBS, and basic fibroblast growth factor (bFGF) in the media, or DM with FBS and bFGF contained in the aPA scaffold. By EdU staining (Fig. 5C) we observed that the encapsulation of GFs (FBS and bFGF) (43, 44) significantly increased the frequency of proliferating primary myoblasts (>60%) compared with DM alone (≈30%), but had similar effects to GFs in the media after 1 d in culture (Fig. 5D). We then changed all media to DM alone and observed that proliferation dropped in all groups at day 3 (Fig. 5D). These results suggested that encapsulated GFs were active on primary myoblasts but that they also diffused into the surrounding media.

To further investigate the dynamics of protein retention and release in aPA gels, we encapsulated 100 ng of vascular endothelial growth factor (VEGF) and 250 ng of bFGF in 10 μL of mid G′ aPA solution and injected the resulting mixture into 1 mL of two different media (Fig. 5E). We then collected and replaced these media and tested both VEGF and bFGF release and retention by ELISA in the media and inside the gel, respectively (Fig. 5F). We found that both VEGF and bFGF were exceptionally well retained in media that did not contain protein over the course of 16 d (i) and that most of VEGF and bFGF were found inside the aPA gel (Fig. 5F). However, we observed significant release of both VEGF and bFGF in media that contained 5 g/dL of the “competing” protein BSA over the course of 16 d (ii) (Fig. 5F). This concentration of BSA is equal to that of the total

Fig. 5. Proliferation and differentiation of primary myoblasts in aPA scaffolds and GF encapsulation and release by aPA scaffolds in vitro. Primary myoblasts were encapsulated at a density of 10,000 cells per microliter. (A and C) Representative confocal micrographs of primary myoblasts in the mid G′ aPA scaffold at different time point culture conditions. Proliferating cells were stained for EdU incorporation (green) and total nuclei were stained with 7-AAD (red). Myosin heavy chain was stained using the MF20 antibody (A, blue). (Scale bar, 100 μm.) (B and D) Graph showing the quantification of EdU incorporation (24-h incubation), from the images in A and C (mean ± SEM). (C and D) After the first day in culture, all media were changed to DM alone. **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, nonsignificant; one (D) or two-way (B) ANOVA with Bonferroni post hoc test. (E) Schematic representation of the protein encapsulation and release experiment. (F) Cumulative release (Left), and amount retained (Right), of VEGF and bFGF from mid G′ aPA gels in two different media, expressed as percentage of total (released + retained) (mean ± SEM).
amount of serum protein found in common mouse strains, such as C57BL/6 (45). Hence, these results suggested that the mid G′ aPA gels would be able to retain and release GFs over the course of tissue regeneration in physiological conditions.

**Biomimetic Scaffolds Loaded with GFs Enhance Engraftment of Transplanted MuSCs.** We evaluated whether biomimetic scaffolds improved MuSC transplantation therapy using a transgenic transplantation model. Primary MuSCs were isolated by fluorescence-activated cell sorting (FACS) (3, 22) from digested hindlimbs of young adult Gfp/Luciferase double-transgenic mice. Luciferase-activated cell sorting (FACS) (3, 22) from digested hindlimbs of young adult Gfp/Luciferase double-transgenic mice. Luciferase-expressing MuSCs were injected intramuscularly (in a 1-μL volume) into preirradiated immunodeficient NOD/Scid recipients and followed dynamically by noninvasive bioluminescence imaging (BLI), a sensitive quantitative measure of cell engraftment after transplantation (3, 21, 22) (Fig. 6 A). We compared biomimetic scaffolds formed with a myogenic GF mixture (bFGF + FBS) and with or without DMSO, a common carrier used for drug suspension studies. We observed no statistically significant effects of including DMSO so samples were grouped (Fig. 6 B-D). We transplanted a low number (200 cells) of MuSCs per injection, to enable discrimination between injection methods. This number of cells typically results in variable engraftment as reported previously (3), but when delivered with a biomimetic scaffold, the variability was greatly reduced and overall engraftment success was substantially improved, attesting to its beneficial effects (Fig. 6 B-D).

In unjured recipients, GF-laden biomimetic scaffolds substantially enhanced MuSC engraftment and donor-cell-mediated myofiber repair posttransplant, due to expedited expansion within 2 wk (Fig. 6 B) and a more frequent successful engraftment outcome at 5 wk (Fig. 6 C and D). Immunohistochemical analysis of donor (GFP′)-derived cells showed enhanced transplanted cell contributions to mature myofibers at the completion of the repair process by MuSCs encapsulated in GF-laden biomimetic scaffolds compared with cells injected in buffer/GF mix (Fig. 6 E and F).

We observed a similar engraftment improvement in primary myoblast (progenitor) transplants. Primary myoblasts were derived from Gfp/Luc mice following culture and expansion and were injected in GF-laden biomimetic scaffolds. We transplanted 3,000 myoblasts, a cell number that results in infrequent engraftment owing to posttransplant myoblast death and differentiation (3, 22) (SI Appendix, Fig. S16A). Relative to buffer controls, we observed enhanced myoblast engraftment and proliferation in transplants with the aPA scaffold (SI Appendix, Fig. S16 B–D). The scaffolds improved myoblast survival as seen by comparing the fraction of transplants with detectable engraftment at 5 wk posttransplant (SI Appendix).

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**Fig. 6.** Biomimetic scaffolds enhance MuSC transplantation therapy. (A) Primary mouse muscle stem cells were isolated from Gfp/Luciferase double-transgenic mice by GFP′/CD34′/integrin-α7′ FACS sorting and mixed with 13 mM mid G′ aPA solution containing bFGF and FBS (+GF′) or no serum/growth factors (−GF′); in G and H only) at 200 cells μL−1. Biomimetic scaffold/MuSC mixtures (1 μL per muscle) were extruded into the TA muscles of preirradiated NOD/Scid by intramuscular injection to form biomimetic scaffolds in situ. In contralateral hindlimbs, control MuSC injections were performed in resuspension buffer/±GFs. Injections were performed with or without DMSO (1.8% final) to evaluate the effect of carrier in drug resuspension studies. No statistically significant effects between control (DMSO-free) and DMSO condition were observed for any comparison so n = 10 samples were grouped per method. Some hindlimbs were injured by intramuscular injection of notexin 3 d pretransplant in G. (B–F) BLI and immunohistochemical detection transplanted MuSC engraftment and myofiber repair in uninjured muscles. Engraftment threshold (dashed line) corresponding to histological detection of one or more donor-derived (GFP′) myofibers (as in refs. 21 and 22). (B) BLI normalized to injected cell number at 0–5 wk posttransplant of n = 10 control (five control, five DMSO) transplants grouped by injection method (p, photons). **P < 0.0001 by two-way ANOVA with Bonferroni post hoc test for comparison of time courses. (C) Normalized BLI values at 5 wk posttransplant. Scatterplot overlain on box (50%) and whisker (full range) with median line. **P < 0.01 by Mann–Whitney U test on confidence intervals of endpoints. (D) Engraftment analysis using threshold BLI value. **P < 0.01 by Fisher’s test on endpoint values. (E and F) Detection of transplant-derived (GFP′) myofibers by anti-GFP and anti-laminin immunohistochemistry from DMSO-free transplants. (E) Representative immunohistochemical images. (Scale bar, 500 μm.) (F) GFP′ myofibers per recipient TA muscle (median line). n = 4 transplants per method. (G and H) BLI detection (5 wk posttransplant) of MuSC engraftment into either uninjured (H) or notexin preinjured hindlimb muscles (G) via biomimetic scaffold encapsulation, with and without bFGF/FBS-loading but not DMSO. Scatterplot shows n = 4 transplants with condition with median line. In F–H, *P < 0.05 by Mann–Whitney U test. ns, not significant.
Importantly, myoblast transplantation was successful with GF-laden biomimetic scaffolds even in the absence of chemically or mechanically induced muscle injury, which is often used to enhance the regenerative function of transplanted myogenic cells (3, 6, 17, 22).

To more closely examine the effects of muscle injury, we performed MuSC transplants into recipient muscles previously injured with the snake venom toxin notexin, a standard injury paradigm that induces extensive damage throughout the injected muscle, together with scaffolds with and without GFs (Fig. 6G). Notably, in injured muscles, the GF inclusions were not required as the GF-free scaffolds markedly improved MuSC engraftment compared with buffer (−GF) controls (Fig. 6G). We hypothesized that injured muscles may transiently generate sufficient prosurvival signals from infiltrating macrophages (46–50) that act in synergy with the GF-free scaffolds, enhancing MuSC engraftment. Hence, we reasoned that GFs codelivered within aPA scaffolds might improve MuSC engraftment and overcome the need for notexin injury in the standard injection paradigm, allowing for a minimal injury approach more favorable to clinical application (Fig. 6H). We observed that, in contrast to MuSC delivery with buffer or scaffold alone, in which cells did not engraft, the GF mixture was required for the marked scaffold-enhanced MuSC transplants engraftment in uninjured recipients (Fig. 6H). Taken together, our observations demonstrate that the biomimetic scaffold system enhances engraftment of muscle stem and progenitor cells, and, in conjunction with GFs, can augment their proliferation, differentiation, and function in muscle repair posttransplantation.

**Discussion**

The discovery of murine MuSCs capable of robustly regenerating and restoring strength to injured muscles (1, 3, 7, 16–19), and a means for their expansion in culture using inducers such as p38 MAPK inhibitors, has provided a framework for their future clinical application (22, 51). We observed that, in contrast to MuSC delivery with buffer or scaffold alone, in which cells did not engraft, the GF mixture was required for the marked scaffold-enhanced MuSC transplants engraftment in uninjured recipients (Fig. 6H). Taken together, our observations demonstrate that the biomimetic scaffold system enhances engraftment of muscle stem and progenitor cells, and, in conjunction with GFs, can augment their proliferation, differentiation, and function in muscle repair posttransplantation.

**Materials and Methods**

We generated PAs consisting of an aliphatic (C16) palmitoyl tail and a hydrophilic six or nine amino acid cap using a custom peptide synthesizer. We exposed the amino acid cap and internally aggregate the hydrophobic tail, by entropic mixing. We extruded aPAs into physiological calcium concentrations in culture medium to fabricate a stable, noodle-like aPA scaffolds with liquid crystalline properties due to their highly ordered nanofibers in parallel to the direction of extrusion. We measured the storage modulus of aPA scaffolds using shear rheometry and AFM. We evaluated myogenic progenitor cell viability, proliferation, and differentiation after encapsulation in assembled aPA scaffolds in vitro and growth differentiation media. We transplanted FACS-isolated α7-integrin+ CD34+ muscle stem cells from GFP/Luciferase transgenic mice mixed with a 13-mM aPA solution by rate-controlled injection into hindlimb muscles of irradiated immunodeficient NOD/scid mice and measured muscle repair by bioluminescence imaging and anti-GFP immunohistochetry at 1-mo posttransplantation. aPA scaffold degradation in vivo was assessed using Gd(III)-doped PA molecules and MRI imaging and ICP analysis every 5 d. Please refer to SI Appendix for details.

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Supporting Information

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Movie S1. Three-dimensional reconstruction of C2C12-GFP cells in nonaligned or aligned mid G′ aPA scaffolds imaged by confocal microscopy. Live cells were stained with Calcein AM (green) and aPA was stained with propidium iodide (red).

Movie S2. Illustration of how our injection system was used to place a string-like scaffold (blue) by injecting 1 µL of aPA solution into the tibialis anterior (red structure) of mice (not shown for clarity). Once the needle was positioned inside the tibialis anterior, the syringe was retracted at a speed of 2 cm/min using a leadscrew linear actuator. The syringe plunger was fixed in place to allow the retracting syringe to move into the stationary plunger.

Other Supporting Information Files

SI Appendix (PDF)
SI Appendix

Author Contributions

E.S. designed and performed, and analyzed data of the following experiments: PA synthesis and purification; birefringence imaging; dynamic light scattering and zeta potential; rheology; cell culture; aPA/cell construct formation and growth factor encapsulation; viability analysis; directionality analysis; in vitro proliferation analysis; immunostaining of aPA/cell constructs; protein retention and release studies. E.S. also assisted in the design, performance, and/or analysis of the following experiments: cryogenic transmission electron microscopy; scanning electron microscopy; atomic force microscopy; MRI; quantification of Gd(III) concentration using inductively coupled mass spectrometry (ICP-MS); transplantation of cells and aPA biomimetic scaffolds. E.S. wrote the manuscript.

B.D.C. designed and performed, and analyzed data of the following experiments: muscle stem cell and primary myoblast isolation; transplantation of cells and aPA biomimetic scaffolds; assessment of muscle cell engraftment and BLI and tissue immunohistochemistry. B.D.C. wrote the corresponding parts of the manuscript and edited it.

M.T.M. designed and performed, and analyzed data of the following experiments: scanning electron microscopy; injection of cells and aPA solutions with an automated injection apparatus; TEM embedding, sectioning and imaging; transplantation of cells and aPA biomimetic scaffolds. M.T.M. also assisted in the design, performance, and/or analysis of the following experiments: rheology, MRI; quantification of Gd(III) concentration using inductively coupled mass spectrometry (ICP-MS). M.T.M. wrote the corresponding parts of the manuscript.

A.T.P. designed and performed, and analyzed data of the following experiments: MRI; quantification of Gd(III) concentration using inductively coupled mass spectrometry (ICP-MS) and TEM imaging. A.T.P. wrote the corresponding parts of the manuscript.

M.H.S. designed and performed, and analyzed data of the following experiments: atomic force microscopy. M.H.S. wrote the corresponding parts of the manuscript.

C.C. and C.R.P. designed and performed, and analyzed data of experiments of: cryogenic transmission electron microscopy.

R.D.H. performed experiments of: transplantation of cells and aPA biomimetic scaffolds; assessment of muscle cell engraftment by BLI.

T.J.M., H.M.B. and S.I.S. supervised the project, proposed and devised experiments, analyzed experiments and edited the manuscript.

All authors accepted the final version of the manuscript.
**Extended Materials and Methods**

**PA synthesis and purification.** All PAs except the Gd(III)-labelled PA (1) were synthesized in the Simpson Querrey Institute’s Peptide Synthesis Core at Northwestern University. PA synthesis was carried out using a CEM Liberty microwave-assisted peptide synthesizer via standard 9-fluorenly methoxycarbonyl (Fmoc) solid-phase peptide synthesis on rink amide MBHA resin. PAs were purified by reverse-phase HPLC on a Varian Prostar 210 HPLC using a water/acetonitrile (each containing 0.1% v/v ammonium hydroxide) gradient. Eluting fractions containing the desired PA were confirmed by mass spectrometry using an Agilent 6520 LCMS. Confirmed fractions were pooled and the acetonitrile was removed by rotary evaporation before freezing and lyophilization. The purity of lyophilized products was tested by LCMS using an Agilent 6520 LCMS. After lyophilization, PAs were dissolved at ~1% weight/volume in mQ water and pH was adjusted to ~7 with NaOH, making PAs completely soluble. These solutions were then dialyzed against mQ water, aliquoted in cryotubes, and lyophilized again. These lyophilized PA aliquots were then soluble when resuspended in water or various aqueous buffers. The Gd-aPA mixture was prepared by adding 5 volumes of a 13mM of the Gd(III)-labelled PA to 95 volumes of a 13mM solution of the mid G’ PA and then annealing (see “Rheology”).

**Cryogenic transmission electron microscopy (CryoTEM).** In one set of experiments, PA solutions (4.6.5 µL) were deposited onto 300-mesh lacey carbon grids (Electron Microscopy Sciences) inside a FEI Vitrobot Mark IV, which maintained 95% during blotting. Lacey carbon grids were glow-discharged for 30 seconds prior to use, using a PELCO easiGlow system (Ted Pella, Inc., Redding, CA, USA). Blotted samples were plunged into liquid ethane and transferred to liquid nitrogen. Samples were imaged using a JEOL 1230 TEM operating at 100kV, inside a Gatan 626 cryo-holder. In another set of experiments, PA samples were plunged frozen using a Vitrobot Mark IV (FEI, Hillsboro, OR) operating at 25°C with 100% humidity. The PA sample (8 µL) was deposited on 300 square mesh copper grids with a lacey carbon film (Ted Pella, Redding, CA), blotted, and plunged into a liquid ethane reservoir cooled by liquid nitrogen. Following vitrification, the sample was transferred to a Gatan 626 cryo-holder (Gatan, Pleasanton, CA) under liquid nitrogen with the aid of a transfer stage. Images were obtained using a Hitachi HT-7700 Biological TEM (Hitachi High Technologies America, Schaumburg, IL) equipped with a LaB6 filament working at an accelerating voltage of 100 kV and an Orius SC 1000A CCD camera.

**Birefringence imaging.** All images were taken with a digital camera (Sony) attached to the eyepiece of a Nikon Eclipse 2000 microscope equipped with cross polarizers. Samples of annealed or non-annealed PA solutions were deposited as 2-µL droplets on top of a glass slide and promptly imaged. PA gel scaffolds alone or with cells (C2C12-GFP) were made by extruding aPA solutions into a gelling solution (120 mM NaCl, 20 mM CaCl2 in mQ water) on top of a glass slide and promptly imaged. Images were scaled according to 45-µm polystyrene beads (Alfa Aestar, CAS# 9003-53-6) imaged in the same conditions.
Scanning electron microscopy (SEM). Samples were fixed in a 3% glutaraldehyde solution for 20 min then dehydrated in a series of ethanol/water washes of increasing ethanol concentration (between 30% and 100%). When samples reached 100% EtOH they were then critically point dried using an automated Samdri®-795 (Tousimis), mounted with carbon adhesive tape, and coated with approximately 8 nm of osmium before imaging using a LEO Gemini 1525 sFEG SEM instrument.

Dynamic light scattering (DLS) and zeta potential measurements. Measurements were performed using a Zetasizer Nano ZSP instrument (Malvern) using disposable folded capillary cells (#DTS1070, Malvern). The different PAs at 13 mM in resuspension solution (150 mM NaCl, 4 mM KCl, in milli Q water) were diluted 1:100 to 0.13 mM in tris-buffered saline (TBS, 20 mM Tris, 136 mM NaCl) and loaded into the capillary cells. A size measurement was performed followed by a zeta potential measurement. For size measurements, the material was considered to be protein with a refractive index (RI) of 1.45, the dispersant was considered to be water with a RI of 1.33, the temperature was set at 25°C, the measurement angle was 173° (default), the measurement duration was set on automatic, each sample was measured 5 times, “extend duration for large particles” was not selected, the positioning method was set to “seek for optimum position”, and automatic attenuation was selected. For zeta potential measurements, the sample was considered to be protein with an RI of 1.45, the dispersant was considered to be water with a RI of 1.33, the temperature was set at 25°C, the model used was Smoluchowski, the measurement duration was set on automatic, each sample was measured 5 times with a delay of 30 seconds between measurements, and both automatic attenuation and automatic voltage were selected.

Rheology. PAs aliquots were dissolved in resuspension buffer (RB, 150 mM NaCl, 4 mM KCl in sterile mQ water) to generate 13 mM solutions with a pH range from 6.8 to 7.4. These solutions were then annealed by placing them in a ~10 liter water bath at 80°C for 30 minutes and then turning off the water bath to let the samples cool down overnight. The slow cooling and the presence of salts are essential to generate long nanofibers in solution (2). Rheological measurements were carried out in an Anton Paar MCR 302 instrument using a conical plate with a 106-µm gap (CP25-2). In order to measure gel stiffness immediately after gelation, annealed PA (aPA) solutions (150 µL) were placed on the lower plate and droplets of a 200 mM CaCl₂ solution (adding up to 20 µL) were evenly distributed on the top plate. Hence, once the top plate was lowered to the measuring position, gelation could be initiated by calcium ions. After this procedure, all gels appeared uniform in composition without any evidence of phase separation. During all experiments the stage temperature was maintained at 23 °C by a Peltier heating system, and samples were isolated from the environment with mineral oil to minimize water evaporation. A 20-minute time sweep was performed at 10 rad/s and 0.1% strain, followed by a strain sweep from 0.1% to 100%. The values reported in Fig. S9 are the average ± SEM of all G’ values from the frequency sweep.

Atomic force microscopy (AFM). The elastic modulus of the gels was measured on a Bioscope Resolve Atomic Force Microscope (Bruker Corp.) in indentation mode. An AFM cantilever with a ~800 nm diameter spherical SiO2 bead attached to the tip and a
30 nm gold coating (Novascan Technologies, Inc) was used in liquid. The deflection sensitivity of the cantilever was calibrated on a glass coverslip, and the spring constant was measured by the thermal noise method to be \( k = 0.085 \) N/m (3). 1.5 \( \mu L \) droplets of each peptide amphiphile solution were deposited on a Poly-D-Lysine (PDL) coated petri dish to ensure immobility of the sample during indentation, and gelling solution was added to the dish until the droplet was submerged. Oriented gels were prepared as described previously, and then deposited on PDL coated dishes. All indentation measurements were performed in a bath of gelling solution. The indentation was conducted at a rate of 5 \( \mu m/s \), up to a maximum deflection force set point of 1 nN, at different locations on the surface of the gels. The spherical Hertz model was used to fit the deformation measured during the loading curve, and calculate the elastic modulus (4). Statistical analysis was performed in Prism 5 (GraphPad).

**Cell culture.** Both C2C12-GFP (C2C12 cells retrovirally transduced and routinely purified by flow cytometry for GFP expression) and mouse primary myoblasts (from C57BL/6J strain) were cultured as described previously (5). Both cell types were plated onto collagen I-coated plates. The collagen solution was prepared as follows: we placed 895 ml of distilled water into a beaker; we then added 5.15 ml of concentrated acetic acid into the water; we then filter-sterilized the solution with a 0.45 \( \mu m \) 500 ml filter inside a culture hood and placed 450 ml into each of two sterile bottles; finally, we added 10 ml of the stock collagen type I solution from calf skin (Sigma, #C8919), mixed it, and stored it at 4°C until use. Dishes were coated as follows: we poured enough collagen I solution into a dish to generously cover the entire bottom of the dish; we then kept the dish overnight in a cell culture hood or kept it at 37°C for 4 hours; we then aspirated off the collagen solution, added a generous amount of sterile water or PBS to the dish, and aspirated again. Collagen I-coated dishes were stored at RT and used within a month. C2C12-GFP cells were cultured in growth medium (GM1) made of 79% high glucose DMEM (ThermoFischer, #10566), 20% FBS (Thermo Fisher, #10082147), 1% penicillin-streptomycin (Thermo Fisher, #15140-122), and 2.5 ng/ml bFGF (Peprotech, #AF-100-18B); and they were passaged at \( \approx 1:10 \) once 75% confluency was reached. Primary myoblasts were passaged in growth medium (GM2) made of 42% low glucose DMEM (ThermoFischer, #10567), 42% F10 nutrient mix (ThermoFischer, #11550), 15% FBS, 1% penicillin/streptomycin, and 2.5 ng/ml bFGF. They were passaged at \( \approx 1:10 \) once 15% confluency was reached. In some experiments, C2C12 cells were also cultured in differentiation media (DM1) composed of 97% high glucose DMEM, 2% horse serum (Sigma, #H1270) and 1% penicillin/streptomycin. Primary myoblasts were also cultured at the indicated times in differentiation media (DM2) composed of 97% low glucose DMEM, 2% horse serum and 1% penicillin/streptomycin.

**aPA/cell construct formation and GF encapsulation in vitro.** On the day prior to aPA/cell construct formation, aliquots of lyophilized PAs (see “PA synthesis and purification”) were dissolved in an appropriate volume of RB to generate 13 mM solutions (all PA solutions had \( \text{pH} \) values in the range 6.8 to 7.4). These solutions were then annealed (see “Rheology”) and used the next day to mix with and encapsulate C2C12-GFP or primary myoblast cells in subsequently formed string-shaped hydrogels. Cells were washed once with PBS and lifted off by incubation with 0.05% trypsin-EDTA.
(Thermo Fisher, #25300-054) at 37°C. The same volume of soybean 0.025% soybean trypsin inhibitor (Sigma, #T6522) was used to neutralize the trypsin. Then, two volumes of GM were added and the cells were resuspended and placed in 1.5 ml tubes before being centrifuged at 400g for 5 minutes. Cell pellets were then washed once with 1 ml of PBS and centrifuged again. Cell pellets were resuspended in 5 µL with PBS, and at this point 25 µL of aPA solution was added to each cell suspension. The resulting 30 µL of aPA/cell suspension was mixed well by pipetting. Hence, the final PA concentration in aPA/cell suspensions was ~10.8 mM. Aligned aPA/cell constructs were made by extruding 4-5 µL onto a sterilized glass slide with 1-2 ml of filter-sterilized gelling solution (120 mM NaCl, 20 mM CaCl2 in mQ water) inside a cell culture hood. We prepared non-aligned aPA/cell constructs by depositing aPA/cell solutions into string-shaped molds and adding gelling solution on top of them. Aligned and non-aligned aPA/cell scaffolds were then picked up with tweezers and placed in a tissue culture dish with GM (except for GF encapsulation experiments). For experiments with GF encapsulation, cell pellets were resuspended in either 5 µL of PBS or in 4.5 µL of FBS and 0.5 µL of a 50 µg/ml solution (25 ng total) of bFGF (Peprotech, #AF-100-18B) and then mixed with 25 µL of aPA solution. All the aPA/cell solution was used to make scaffolds and they were then placed in dishes containing 5 ml of GM (positive control), DM alone (for negative control and GFs encapsulated in the aPA), or DM containing the same amount of encapsulated FBS (4.5 µL) and bFGF (25 ng). Hence, the final concentration of GFs encapsulated by the aPA scaffold were 15% FBS and 833 ng/ml bFGF; but, if all the GFs diffused into the media, the final concentration of GFs in the media would be 0.09% FBS and 5 ng/ml bFGF. For viability and directionality experiments, aPA/cell constructs were cultured for one day in GM and then cultured in DM thereafter.

Viability analysis. At different times in culture, samples of aPA/cell constructs were collected using a wide-bore tip (Axygen, #14-222-766) and placed in PBS containing 2.5 µL/ml of a 1 mg/ml Calcein AM solution (Thermo Fisher, #C1430) and 100 ng/ml propidium iodide (PI, Sigma, #P4170) at 37°C for ~15 min. The aPA/cell constructs were then mounted in PBS on a glass slide to which a rectangular frame of vacuum grease was made in order to keep the 3D structure of the aPA/cell constructs once the coverslip was placed on top. Confocal images were then immediately taken at the Center for Advanced Microscopy at Northwestern University using a Nikon A1R+ Confocal Laser Microscope System or a Zeiss UVLSM 510 Meta microscope. Files were then opened in Fiji (6) and live (Calcein AM-positive) and dead (PI-positive) cells were counted using the “analyze particles” function using a custom protocol. For C2C12s, 5-6 images from each sample and time point from 2 independent experiments were analyzed. For primary myoblasts, >500-1100 live cells were counted in 3-5 images from one experiment.

Directionality analysis. Confocal images used in the viability analysis (Calcein AM channel) were opened in Fiji (6) and the “Directionality” plugin (http://fiji.sc/Directionality) was applied using the “local gradient orientation” method with the “display table” option checked. The table generated to build the directionality histogram by the plugin containing the values of direction, their amount, and the values of the associated
Gaussian function fit were imported into Excel. Then the maximum and minimum values from the Gaussian function fit were found. By dividing the maximum by the minimum, we obtained a measure equivalent to a signal-to-noise ratio that we called “order parameter”. An order parameter of 1 means that cells do not align with any preferred direction but the higher this value is, the more preferred a direction becomes and hence more aligned the cells are to each other. All values obtained from the Directionality plugin and Excel were imported into Prism for statistical analysis and generation of custom histograms. For each sample and time point, 5-9 images were analyzed from 2 experiments.

**In vitro proliferation analysis.** To stain for proliferative cells, 2 µM of the thymidine analog 5-ethyl-2'-deoxyuridine (EdU, Thermo Fisher, #A10044) was incorporated into the culture medium for 24 hours before PA/cell constructs were fixed at the indicated time points in 4% paraformaldehyde (PFA) for 1hr at room temperature (RT) or overnight at 4°C. After fixation samples were stained as described (7, 8). Briefly, aPA/cell constructs were washed in PBS twice and then stained with EdU staining cocktail containing 100 mM Tris (from 2M stock, pH 8.5), 1 mM CuSO4 (from 50 mM stock), 100 mM ascorbic acid (from 500 mM stock) and 1 µg/ml of Alexa Fluor azide (from 0.5 mg/ml, Thermo Fisher, #A10266, #A20012) in mQ water for 30 minutes at RT in the dark. The staining cocktail was then removed and aPA/cell constructs were washed with PBS. At this point, aPA/cell constructs were used for further immunostaining (see below) or counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Thermo Fisher, #D1306) at 5 µg/ml or 7-aminoactinomycin D (7-AAD, Thermo Fisher, #A1310) at 0.5-1.25 µg/ml in PBS overnight at 4°C, washed with PBS, and mounted with Immu-Mount (Fisher Scientific, #9990402). Confocal images were taken at the Center for Advanced Microscopy at Northwestern University on a Nikon A1R+ Confocal Laser Microscope System. Image files were imported into Fiji (6) and the “analyze particles” and “cell counter” functions were used to measure the number of total nuclei (DAPI or 7-AAD positive) and proliferative nuclei (EdU positive). For C2C12s, >500-2000 total nuclei were analyzed from 5-6 images in the first experiment and >750-6000 from 17-36 images in the second experiment. To weigh the contributions of each experiment equally, we plotted the means for the conditions that had repeated experiments. For primary myoblasts, >100-300 total nuclei were analyzed from 5-13 images from 2 experiments (in DM conditions) and 400-3,000 total nuclei were analyzed from 5-9 images from one experiment (in GM conditions).

**Immunostaining of aPA/cell constructs.** After EdU staining (see above), if applicable, fixed aPA/cell constructs (see above) were then blocked with 0.5% triton X-100 (Sigma-Aldrich, #X100) and 10% normal goat serum (NGS, Thermo Fisher, #16210-064) in tris-buffered saline (TBS) for 1 hour at RT. The antibody against alpha-actinin (Sigma, #EA-53) or the MF20 antibody (DSHB, #MF20, supernatant), which recognizes all myosin heavy chain (MHC) isoforms, were used at a 1:100 dilution in 0.1% triton X-100 and 10% NGS in TBS (TBS++) and incubated for 5 days at 4°C rocking. No antibody against green fluorescent protein (GFP) was used. aPA/cell constructs were then washed 3 times in TBS++ for 10 minutes each at RT and then incubated with an anti-Mouse IgG secondary antibody (Thermo Fisher, #A-31553) and PI or 7-AAD at 4-5 µg/ml, 100
ng/ml, or 0.5-1.25 µg/ml, respectively, in TBS++ for 2 hours at 37°C or overnight at 4°C. Finally, aPA/cell constructs were washed in TBS++ and TBS and mounted with Immumount.

**Protein retention and release studies.** 1 µL containing 100 ng of VEGF (Peprotech, #AF-100-20) and 1 µL containing 250 ng of bFGF (Peprotech, #AF-100-18B) were placed in a 1.5 ml tube and mixed with 10 µL of 13 mM mid G’ aPA. The resulting 12 µL solution was pipetted into wells of a 24 well-plate containing 1 ml of filter-sterilized media consisting of TBS with 2 mM CaCl₂ or consisting of DMEM with 5 g/dL of BSA (Sigma, #A94189), resulting in the formation of string-like gels. The plate was then placed in a 5% CO₂ incubator at 37°C for 16 days and the full 1 ml of media was collected and replaced at days 1, 3, 7 and collected at day 16. All media were stored at 4°C until used. After media collection at day 16, the gels were dissolved in a solution containing 50 mM tris pH 8.2, NaCl 300 mM, 0.02% NaN₃, 5 mM EDTA, 1% triton X-100 and 1x Halt proteinase inhibitor cocktail (ThermoFisher, #87786). The human VEGF and FGF-basic mini ELISA development kits (Peprotech, #900-M10 and #900-M08, respectively) and ELISA buffer kit (Peprotech, #900-K00) were used to determine VEGF and bFGF concentrations in the media and inside the gel, according to manufacturer’s instructions. The total amount of measured VEGF and bFGF were obtained by adding the released amounts of all days analyzed to what was left inside the gel. We then represented the cumulative percentage amounts of released protein at each time point together with the percentage amount of retained protein inside the gel.

**Injection of cells and aPA solutions with an automated injection apparatus.** For all transplants, cells were resuspended in PBS buffer or mid G’ aPA solution. 1 µL of these solutions or the Gd(III)-labeled aPA mixture were injected at a rate of 3 µL/min into the TA muscle as a ~0.9 cm long tract. The needle translational movement, 2 cm/min, was controlled with a modified NE-300 Just Infusion Syringe Pump, setup shown in Fig. 4. This set up ensured that the ratio of the exiting aPA velocity and retracting needle velocity remained ~1 and constant. We were able to keep these two values equal by using a needle inner diameter that matched the Hamilton syringe’s inner barrel diameter, ~0.5 mm (10 µL Hamilton Syringe and 21G needle) (Fig. S5). When the needle’s inner diameter was too small relative to that of the syringe barrel, we observed the non-uniform accumulation of the fluid scaffold beyond the boundaries of the needle tract (Fig. S5). When this happened, we observed that the long-range alignment of nanofibers was compromised, possibly due to the extreme sensitivity of liquid crystals to mechanical forces. At the same time, a very slight enlargement of the needle diameter relative to the barrel leads to some degree of extensional flow in the fluid scaffold as it contacts muscle tissue. We reasoned that the extensional flow would insure the macroscopic alignment of the very long nanofibers throughout the entire injected scaffold. This flow is the result of the greater velocity of the retracting needle relative to the exit velocity of the fluid scaffold (approximately 12% difference). Our delivery method couples the retracting needle motion to fluid flow rate, thus enabling a constant ratio of these two velocities. Once injected, ions present in the tissue gelled the aPA solution localizing it at the site of injection.
**TEM embedding, sectioning and imaging.** For TEM sample preparation, mice were injected with 10.4 mM mid G’ aPA loaded with Evans Blue dye for visualization. Immediately following injection, mice were sacrificed. The legs were removed and skinned prior to fixation in phosphate buffered (0.1M, pH 7.4) 4% paraformaldehyde at 4°C for 24 h. After fixation, the skinned legs were dissected into approximately 3 mm x 3 mm x 1 mm cubes. The presence of the aPA scaffolds in these cubes could be visualized by the presence of Evan’s blue dye. These small cubes were transferred to modified Karnovsky’s fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h at RT. Samples were post-fixed in osmium tetroxide and stained en bloc by uranyl acetate solution. Stained samples were then dehydrated by incubation in a graded series of ethanol. Following dehydration, ethanol was replaced with propylene oxide, and finally the samples were embedded in resin (Embed 812, Electron Microscopy Sciences) through intermediate exchange steps. 70-nm sections of resin-embedded muscle were prepared (sectioned 185 parallel to the direction of aPA scaffold long-axis) using a Leica Ultracut UCT ultramicrotome. Sections were post stained with lead citrate and uranyl acetate for enhanced contrast and micrographs were obtained on a JEOL 1230 TEM microscope operating at 120 kV.

**MRI.** All images were acquired on a Bruker 7T Pharmascan MRI system using a 38 mm quadrature coil. Anatomical images were acquired with a fat-saturated multispin multiecho (MSME) pulse sequence (TR/TE = 800 ms/11.5 ms) with 5 slices of 1 mm thickness and two signal averages. The field of view was 4 x 4 cm with a 300 x 300 pixel matrix size. T1 maps for the animal experiments were acquired using a rapid acquisition with relaxation enhancement (RARE)-based T1 map protocol with repetition times 157, 200, 400, 800, 1200, 3000, and 4000 ms with one average and no fat saturation. T2 acquisitions were taken using a fat-saturated MSME T2 map sequence with repetition time 4000 ms and echo times 11.6, 23.3, 34.9, 46.5, 58.2, 69.8, 81.4, 93.1, 104.7, and 116.4 ms. Slice geometry parameters for T1 and T2 maps were the same as above. T1 maps were generated with a saturation-recovery fit for each pixel using the Jim software package (Xinapse Systems, Colchester, UK).

**Quantification of Gd(III) concentration using inductively coupled mass spectrometry (ICP-MS).** Whole mouse TA's were manually dissected from mice at four time points and stored at -80°C until analysis, n ≥ 4. TA samples were then digested using ACS reagent grade nitric acid (70%) for a least 2 hours at 70°C. Samples were then diluted in filtered, deionized water. A standard containing the elements Bi, Ho, In, Li, Sc, Tb, and Y was added to a final concentration of 5 ng/mL (Inorganic Ventures, Christiansburg, VA, USA) (the final nitric acid concentration was 3%). The instrument was calibrated using a serial dilution of Gd (III) standards (Inorganic Ventures, Christiansburg, VA, USA) with nitric acid and internal standard concentrations identical to the samples. Calibration was conducted with 1, 5, 10, 20, 50, 100, and 200 ng/mL Gd(III) standards. For Gd(III) analysis, mouse leg muscles were excised and digested in 1 mL of nitric acid per gram of tissue in Teflon tubes. A Milestone EthosEZ microwave digestion system (Shelton, CT, USA) was used to digest the samples, ramping to 120°C over 30 minutes, then holding at 120°C for an additional 30 minutes. Digest was diluted and measured using the same conditions as above. All measurements used a Thermo
X series II ICP-MS (Thermo Fisher Scientific, Waltham, MA, USA) operating with an ESI SC-2 autosampler (Omaha, NE, USA). Each acquisition consisted of one survey scan, utilizing 10 sweeps, and 3 peak-jumping measurements of 100 sweeps each. The two most common isotopes of Gd, $^{157}$Gd and $^{158}$Gd, were measured. $^{115}$In and $^{165}$Ho were measured as internal standard. Data were imported into Prism and a one-phase decay equation was fit to the data.

**Animals.** The Administrative Panel on Laboratory Animal Care (APLAC) for Stanford University or the Institutional Animal Care and Use Committee (IACUC) at Northwestern University approved all animal protocols. We performed all experiments in compliance with the institutional guidelines of Stanford University and Northwestern University. We purchased C57BL/6J mice from Jackson Laboratories. We generated double-transgenic Gfp/Luc mice by crossing C57BL/6J mice expressing a green fluorescent protein (Gfp) transgene under regulation of the ubiquitous UBC promoter (Jackson #6567) with FVB mice expressing a firefly luciferase (Luc) transgene under regulation of the ubiquitous Actb promoter (Jackson #8450), as described previously (9, 10). We validated these genotypes by appropriate PCR-based strategies. For ICP-MS experiments, we injected Gd(III)-labeled aPA solutions into young adult (2–4 months) female C57BL/6J mice. For transplantation experiments, we isolated muscle stem cells or primary myoblasts from young adult (2–4 months) female Gfp/Luc donor mice and transplanted them into young adult (2–4 months) female immunodeficient NOD/Scid recipient mice (Jackson #1303).

**Muscle stem cell and primary myoblast isolation.** We isolated muscle stem cells (MuSCs) by dissociating the tibialis anterior and gastrocnemius muscles of 2–4-month-old female Gfp/Luc mice and enriched for MuSCs by double FACS sorting for GFP+/propidium iodide−/CD45−/CD31−/CD11b−/Sca1−/CD34+/integrin-α7+ cells using a modified FACStar Plus (BD Biosciences), as described previously (10). Primary myoblasts were derived from dissociated muscles of 2-month old female Gfp/Luc mice by pre-plating and culture expansion, as previously described (11). Myoblasts were maintained on collagen I-coated tissue-culture plastic dishes in myogenic cell growth medium, containing 42% low-glucose Dulbecco’s Modified Eagle Medium (DMEM), 42% Ham’s F-10 nutrient mix, 15% fetal bovine serum (FBS), 2.5 ng ml$^{-1}$ basic fibroblast growth factor (bFGF, also known as FGF-2) and 1% penicillin-streptomycin, at 37 °C in 5% CO$_2$. Myoblasts were enriched at passage 4 by FACS sorting for GFP+/integrin-α7+ cells and were used at passage 10–12 for transplantation studies.

**Transplantation of cells and aPA biomimetic scaffolds.** We mixed cells (Gfp/Luc MuSCs or primary myoblasts) with a 13 mM solution of mid G’ aPA yielding a final concentration of ~10.8 mM mid G’ aPA. In some experiments, bFGF (700 ng ml$^{-1}$ final) and FBS (5% final) were added to the aPA/cell mixture. We transplanted cells into the tibialis anterior muscles of pre-irradiated NOD/Scid mice by intramuscular injection via slow extrusion of (1 µL per muscle) the aPA/cell mixture using a 21-G Hamilton syringe controlled by a modified NE-300 Just Infusion Syringe Pump (as described above and in Fig. 3). We delivered 200 MuSCs (in Fig. 5) or 3000 myoblasts (in SI Appendix, Fig. S7) per 1-µL injection as these cell numbers provided good discrimination for engraftment detection for these cell types in prior reports (9, 10, 12). In contralateral tibialis anterior
muscles, we transplanted cells in resuspension buffer without aPA (±bFGF and FBS and ±DMSO). One day prior to transplantation, we anesthetized recipient immunodeficient NOD/Scid mice with ketamine (2.4 mg per mouse) and xylazine (240 μg per mouse) by intraperitoneal injection. We then irradiated hindlimbs with a single 18-Gy dose with the rest of the body shielded in a lead jig, as described previously (10). Some recipient muscles were injured by a single 10-μL intramuscular injection of notexin (10 μg ml⁻¹; Latoxan) 3 days pre-transplant as in (10).

**Assessment of muscle cell engraftment by BLI and tissue immunohistochemistry.** We performed bioluminescence imaging (BLI) using a Xenogen-100 system, essentially as previously described (9, 10), approximately weekly up to one-month post-transplantation. We anesthetized mice using isoflurane and administered 100 μL D-luciferin (0.1 mmol kg⁻¹ in PBS; Caliper LifeSciences) by intraperitoneal injection. We collected BLI output images using a 60-s exposure acquired at 12 min after D-luciferin injection. We analyzed images with Living Image software (Caliper LifeSciences) and applied consistent region-of-interest over each hindlimb to calculate a bioluminescence signal in photons s⁻¹. We normalized these values to the number of injected cells to compare across conditions. We used a BLI signal value corresponding to 80,000 photons s⁻¹ to define an engraftment threshold (dashed line in Fig. 5 and SI Appendix, Fig. S7) as in (10, 12). At one-month post-transplant, we collected recipient TA muscles to immunohistochemically analyze the contributions of transplanted cells to myofiber repair. As previously described (10), we fixed, cryosectioned, and immunostained transverse TA sections. We incubated sections with rat anti-laminin (Millipore, clone A5, catalog # 05-206, 1:250) and rabbit anti-GFP (Life Technologies, catalog # A-11122, 1:200) primary antibodies, and then with TRITC–conjugated donkey anti-rat and AlexaFluor 488–conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, catalog # 712-025-150 and 711-545-152 respectively, 1:200 each) secondary antibodies. We collected images with an AxioPlan2 epifluorescent microscope (Carl Zeiss Microimaging, Thornwood, NY) with Plan NeoFluar 20×/0.75NA objective (Carl Zeiss) and an ORCA-ER digital camera (Hamamatsu Photonics) and captured them in OpenLab software (Improvision). We used Photoshop software (Adobe) to assemble multi-panel images, with consistent contrast adjustments across all images from the same stain, to quantify the number of GFP⁺ myofibers per recipient TA muscle.

**Statistical analysis.** Data were analyzed using GraphPad Prism and a level of α = 0.05 was considered to be significant in all tests. Specific replicate numbers and post-hoc tests used can be found in the figure legends or Materials and Methods section.
Supplementary Figure 1

(a) C16-V2A2E2  C16-V3A3E3  C16-V3A3K3
Non-annealed
Annealed

(b) 

<table>
<thead>
<tr>
<th>Length (nm)</th>
<th>E2 unannealed</th>
<th>E3 unannealed</th>
<th>K3 unannealed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>500</td>
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<td>2000</td>
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Figure S1. PA nanofiber structure and length measurements. a, Cryogenic transmission electron microscopy of 0.13 mM non-annealed or annealed PA solutions (diluted in TBS from the original 13 mM solution). Notice that some nanofibers are too long for their length to be quantified, especially in the annealed samples. Scale bars are 500 nm. b, Fiber length measurements from the images of non-annealed PA in a. Represented are the individual values of all measurements together with their mean (red horizontal line).
Supplementary Figure 2

(a) $C_{16}V2A4E3$

(b) ESI TIC Scan Frag=140.0V ES sample 5.d

(c) ESI Scan (13.0-14.0 min, 143 scans) Frag=140.0V ES sample 5.d Deconvoluted
Figure S2. Chemical structure and purity of C$_{16}$V2A4E3. a, All PA molecules contain a C$_{16}$ aliphatic tail (pink), a β-sheet forming region (shade of grey), and charged amino acids (purple). b, High-pressure liquid chromatography (HPLC) trace of the purified PA. c, Mass spectrometry of the main HPLC peak from b. Note that the mass corresponds to the ammonia (NH$_3$) adduct.
Supplementary Figure 3

a  C_{16}A3V3E3

H N O  H N O  H N O  H N O  H N O  O OH  O OH  O OH

b

DAD1 - A:Sig=220,8 A3V3E3 B1.d

Response Units vs. Acquisition Time (min)

0  2  4  6  8  10  12  14  16  18  20  22  24  26  28  30  32

10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8 10^9 10^10

c

+ESI Scan (13.0-14.2 min, 154 scans) Frag=140.0V A3V3E3 B1.d Deconvoluted

1170,5733

Counts vs. Mass-to-Charge (m/z)

500 1000 1500 2000 2500 3000 3500 4000 4500 5000 5500

1557,7530 2325,1212 3497,7396 4667,2988
Figure S3. Chemical structure and purity of C_{16}A3V3E3. a, All PA molecules contain a C_{16} aliphatic tail (pink), a β-sheet forming region (shade of grey), and charged amino acids (purple). b, High-pressure liquid chromatography (HPLC) trace of the purified PA. c, Mass spectrometry of the main HPLC peak from b. Note that the mass corresponds to the ammonia (NH$_3$) adduct.
Supplementary Figure 4

a $C_{16}V3A3E3$

b DAD1 - A:Sig=220.8 V3A3E3 B3.d

c +ESI Scan (13.6-14.5 min, 123 scans) Frag=140.0V V3A3E3 B3.d Deconvoluted
Figure S4. Chemical structure and purity of $C_{16}V3A3E3$. a, All PA molecules contain a $C_{16}$ aliphatic tail (pink), a β-sheet forming region (shade of grey), and charged amino acids (purple). b, High-pressure liquid chromatography (HPLC) trace of the purified PA. c, Mass spectrometry of the main HPLC peak from b. Note that the mass corresponds to the ammonia ($NH_3$) adduct.
Supplementary Figure 5

(a) $C_{10}V4A2E3$

(b) DAD1 - A: $\text{Sig}=220,8$ V4A2E3 B1.d

(c) +ESI Scan (14.0-14.9 min, 111 scans) Frag=140.0V V4A2E3 B1.d Deconvoluted
Figure S5. Chemical structure and purity of C_{16}V4A2E3. a, All PA molecules contain a C_{16} aliphatic tail (pink), a β-sheet forming region (shade of grey), and charged amino acids (purple). b, High-pressure liquid chromatography (HPLC) trace of the purified PA. c, Mass spectrometry of the main HPLC peak from b. Note that the mass corresponds to the ammonia (NH₃) adduct.
Supplementary Figure 6

(a) $C_{16}V2A2E2$

(b) ESI TIC Scan Frag=140.0V ES sample 3.d

(c) ESI Scan (15.8-16.7 min, 114 scans) Frag=140.0V ES sample 3.d Deconvoluted
Figure S6. Chemical structure and purity of C_{16}V2A2E2. 

a, All PA molecules contain a C_{16} aliphatic tail (pink), a β-sheet forming region (shade of grey), and charged amino acids (purple). 
b, High-pressure liquid chromatography (HPLC) trace of the purified PA. 
c, Mass spectrometry of the main HPLC peak from b. Note that the mass corresponds to the ammonia (NH_{3}) adduct.
Supplementary Figure 7

a
Oriented
Non-oriented

C₁₆V₂A₄E₃
C₁₆V₃A₃E₃
C₁₆V₂A₂E₂
C₁₆V₂A₄E₃
C₁₆V₃A₃E₃
C₁₆V₂A₂E₂

b
Non-annealed PA
13 mM (in 150 mM NaCl, 4 mM KCl)

Annealed PA
0.13 mM in TBS

C₁₆V₂A₄E₃
C₁₆A₃V₃E₃
C₁₆V₃A₃E₃
C₁₆V₄A₂E₃
C₁₆V₂A₂E₂
Figure S7. Physical characteristics of PA gels and solutions. a, Scanning electron microscope images of oriented or non-oriented scaffolds made from aPA solutions. Note that there is a preferred direction for nanofibers on oriented scaffolds while there is not on non-oriented scaffolds. Scale bars are embedded in the images. b, Birefringence images of 13 mM non-annealed PA solutions. Scale bars are 1500 μm and 500 μm for the top and bottom images, respectively. c, CryoTEM images of 0.13 mM annealed PAs (diluted in TBS from the original 13 mM solution). Scale bars are 100 nM.
Supplementary Figure 8

Alone vs w/cells

C16V2A4E3

500 µm

C16V3A3E3

500 µm

C16V2A2E2
Figure S8. Birefringence of cell-seeded and cell-free scaffolds. Birefringence images of “noodle”-shaped scaffolds with (green box) or without (orange box) 20,000 C2C12-GFP cells made with 13 mM aPA solutions. Note that in cell-seeded scaffolds, the loss of birefringence corresponds to the area where the cells are. Scale bars are 500 µm.
Supplementary Figure 9

(a) Bulk gel rheology

Storage modulus ($G'$) vs. Angular frequency (rad/s)

- $G' = 2763 \pm 87.2$ (low $G'$)
- $G' = 9004 \pm 214.7$ (mid $G'$)
- $G' = 14661 \pm 323.5$ (high $G'$)
- $G' = 3614 \pm 80.1$
- $G' = 10226 \pm 778.8$

(b) AFM on non-oriented gels

Young's Modulus (Pa) vs. Gel Type

- low $G'$
- mid $G'$
- high $G'$
**Figure S9. Rheology and AFM of aPA gels.**

*a,* Storage (G’) and Loss (G’”) modulus values as measured by “bulk” gel rheology aPA gels. The aPA gels’ mean ± SEM stiffness (G’) values obtained by averaging all values in the frequency sweep (see “Rheology”) are shown next to the chemical formula (from n = 3-4 measurements per sample). *b,* Young’s modulus values (E) measured on non-oriented aPA gels by atomic force microscopy (AFM). Represented is a box and whiskers plot of the 5-95 percentile (n = 272-768 measurements per sample).
Supplementary Figure 10

mid G’ non-oriented  mid G’ oriented
Figure S10. Alignment of C2C12-GFP myoblasts in oriented or non-oriented mid G’ scaffolds. Confocal stack (left) and scanning electron micrograph (right) of C2C12-GFP myoblasts at day 4 after encapsulation in mid G’ non-oriented or oriented aPA scaffolds. Propidium iodide (red) stains nuclei of dead cells and it also stains non-specifically the aPA fibers. Scale bars correspond to 100 µm and 4 µm for the confocal and SEM images, respectively.
Supplementary Figure 11

(a) Calcein AM images showing the effect of different concentrations of Cytochalasin D on cell morphology.

(b) Graphs illustrating the order parameter for Day 1 (1d GM) and Day 4 (+3d DM) conditions, with raw data and Gaussian fit shown.

(c) Bar graph depicting the order parameter for different concentrations (5k/µl, 20k/µl, 50k/µl) on Day 1 (1d GM) and Day 4 (+3d DM) with statistical significance indicated.

(d) Bar graph showing the order parameter for different Cytochalasin D concentrations (5k, 20k, 50k) across Day 1 (1d GM) and Day 4 (+3d DM) with statistical significance noted.
**Figure S11. Cell density and time dependency of cell alignment.** C2C12-GFP cells were encapsulated in the aligned mid G’ aPA scaffold at different starting cell densities and cultured for 4 days. 

a, Representative confocal micrographs of Calcein AM-stained C2C12-GFP cells in aPA scaffolds at different time points showing differential elongation of cells or cell clusters upon starting cell density and time analyzed (scale bar corresponds to 250 µm). 

b, Directionality analysis from the images in a. The histograms show the angular distribution of the directions in which cells or cell clusters elongate (black bars) and the Gaussian equation that fits the data (red line). The maximum and minimum values of the Gaussian fit are shown and used to calculate the order parameter in c and d. 

c, d, As a measure of directionality, we divided the maximum value of the Gaussian fit by the minimum to calculate an order parameter. When this order parameter is 1, there is no preferred direction in the sample; greater values indicate greater directionality. 

c, Analysis of the order parameter comparing cell densities. 

d, Analysis of the order parameter comparing time points. **p<0.01; ****p<0.0001; ns, non significant; two-way ANOVA with Bonferroni post hoc test.
Supplementary Figure 12

(a) Day 2 (1d GM + 1d DM) and Day 2 (2d GM) shows GFP expression with low G' and mid G'.

(b) Day 2 (2d GM) shows bright field images with low G' and mid G'.
Figure S12. Stiffness-dependent aPA scaffold structure and cell outgrowth. Representative fluorescent (a) or bright field (b) micrographs of C2C12-GFP cells in aligned aPA scaffolds of different stiffness and in different culture conditions. a, Note that low G’ aPA scaffolds are bent while the mid G’ aPA scaffolds retain their original shape. b, Independently of aPA scaffold stiffness, there were outgrowth of cells from inside the aPA scaffold towards the outside culture dish. GM is growth media; DM is differentiation media. Scale bars correspond to 500 µm.
Supplementary Figure 13
Figure S13. a, A macroscopic image of a 1.5-cm aPA scaffold injected into an agarose gel. b, The fluorescently dyed aPA was visualized using a UV lamp. c, Close observations of dyed aPAs being injected into agarose with either a 21G needle or a smaller 23G needle. d, The birefringent pattern resulting from nanofiber alignment was easily viewed through the transparent agarose matrix using polarized light microscopy. The smaller needle results in more disorganized nanofiber channels.
Supplementary Figure 14

Days Post-Injection

2  4  8

Left Leg

Mouse 1

Right Leg

Left Leg

Mouse 2

Right Leg
Figure S14. Examples of $T_1$ maps from $T_1$ measurements (Figure 3) performed at 7T. Each image is one of three $T_1$ map slices. Regions of interest (ROIs) are indicated in red. Square ROIs correspond to background $T_1$ measurement of muscle tissue proximate to point of injection. Free drawn ROIs correspond to PA material. In these images, darker regions denote lower $T_1$ values, and PA localization. No clear PA signal was observed at day 8 in the left leg of Mouse 2. The Circular object appearing in some images is a tube of water added to increase coil fill factor.
Supplementary Figure 15

(a) GM Day 1, GM Day 5, GM (1 day) + DM (4 days)

(b) Viability (% alive)

- GM
- GM (1 day) + DM (4 days)

Day 1, Day 5

ns, **
Figure S15. Viability of primary myoblasts in aPA scaffolds. a, Representative confocal micrographs of primary myoblasts in the mid G’ aPA scaffold at different time points and in different culture conditions stained with Calcein AM (green, alive) and propidium iodide (red, dead). White arrowheads point to dead cells (red dots). Scale bars correspond to 250 µm. b, Viability analysis from the images in a. **p<0.01; ns, non significant; one-way ANOVA with Bonferroni post hoc test.
**Supplementary Figure 16**

**a** Transplantation scheme

- **Gfp/Luc mice**
- **Muscle stem cells**
- **Myoblasts**

In buffer or aPA + bFGF/FBS (GFs)

Myoblasts (3000) in buffer only

Myoblasts in biomimetic scaffold

Extrude via i.m. injection to form biomimetic scaffold in vivo

**b**

Normalized bioluminescence ($10^3$ p s$^{-1}$ injected cell$^{-1}$) vs Days post-transplant

- **Biomimetic scaffold + GFs**
- **Buffer + GFs**

**c**

Bioluminescence images over Weeks post-transplant

**d**

Normalized bioluminescence ($10^4$ p s$^{-1}$ injected cell$^{-1}$) vs Weeks post-transplant

- **Buffer + GFs**
- **Mid G' noodle + GFs**

- Supplementary Figure 16
Figure S16. Primary mouse myoblasts were derived from Gfp/Luc double-transgenic mice and mixed with a 13 mM mid G’ aPA solution containing bFGF and FBS at 3000 cells µL⁻¹. aPA/myoblast mixtures (1 µL per muscle) were extruded into the TA muscles of pre-irradiated NOD/Scid by intramuscular injection to form aPA scaffolds in situ. In contralateral hindlimbs, control myoblast injections were performed in resuspension buffer containing bFGF and FBS but not aPA solution. b, Engraftment was determined by bioluminescence imaging (BLI) with total flux (photons sec⁻¹) at time point normalized by injected cell number (at 0 days). BLI detection of transplanted myoblasts engraftment from 0-5 weeks post-transplant. Mean ± SEM of n = 4 transplants per condition (p, photons). *P<0.05 by two-way ANOVA with Bonferroni post-hoc test for time-course comparison. c, Representative BLI images with BLI radiance at 5 weeks post-transplant (median line). Engraftment threshold (dashed line) corresponding to histological detection of ≥1 donor-derived (GFP⁺) myofibers (as in (10, 12)). *P<0.05 by Mann-Whitney U-test for endpoint BLI confidence interval comparison.
Supplementary References