Supplementary Information

A System to Monitor Statin-Induced Myopathy in Individual Engineered Skeletal Muscle Myobundles

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Materials and Methods

Design and fabrication of the frame, Ecoflex film and mold

The frames were fabricated from nylon (Cerex Advanced Fabrics) using a laser cutter (45 Watt Universal). The Ecoflex films were made by platinum-catalyzed silicones (SMOOTH-ON, Inc., 00-10) with the mix ratio 3:1 (part B: part A). The microbeads (Cospheric, BKPMS-1.2, 53-63 µm diameter) were added at a final concentration of 1.25% (w/w) prior to polymerization. The polymer solution with beads was poured into a Teflon mold (60mm ×12mm ×0.5mm) and covered with a glass slide. Next, the mixture was placed under vacuum for 10 minutes and cured at 60°C for 3 hours to cross-link the Ecoflex. Finally, the Ecoflex was cut into pieces (12mm×3mm×0.5mm or 9mm ×2mm×0.5mm) for attachment to myobundles.

To test whether the elastic force of Ecoflex material was affected by long-term exposure to media, 24 Ecoflex films were divided into 8 groups and each group was soaked in media (day 1-4 in growth media, day 5-35 in shift media) and the force-displacement relation measured at different time points.

There were two designs of mold/frame in this work: one design was the four myobundles (20 µl gel, 4.5mm×1.8mm ×2mm seeding chamber) and Ecoflex films (9mm ×2mm×0.5mm) compacted in the same frame (20.4 × 17.6 mm outer dimensions, 18.6 × 14 mm inner dimensions), which was used in in situ force testing (fig.1a and S1a); The other design was the single myobundle (50ul gel, 6.2mm×2mm ×2.2mm seeding chamber) grown in a smaller frames (19 × 8 mm outer dimensions, 15 × 6.2 mm inner dimensions) and then connected to the Ecoflex membrane (12mm×3mm×0.5mm) (Fig. S1b), which was used in force transducer validation testing.

Human skeletal muscle (HSkM) cells isolation and culture

HSkM samples were obtained using Duke University IRB approved protocols. Myoblasts were isolated as previously described [1]. Briefly, muscle samples were minced, washed with PBS, and digested in 0.05% trypsin (ThermoFisher, 25300062) for 30 min. Then muscle sample was collected by centrifugation, pre-plated for 2 hours, detached, and seeded into a growth factor reduced Matrigel (Corning, 356231) coated flask for attachment and expansion for 4 days. HSkM cells were cultured in skeletal muscle growth media (GM), consisting of low glucose DMEM
(Gibco Life Technologies, 1816871), 8% Fetal bovine serum (FBS, Hyclone, SH30071.03), 0.4 µg/ml Dexamethasone (Sigma, D4902), 10ng/ml EGF (VWR, AF10015), 50µg/ml Fetuin (Sigma-Aldrich, F2379), 1% antibiotic-antimycotic (100X, ThermoFisher, 15240062). The media was changed every other day. The HSkM cells in passage 3-5 were used for generation of myobundles.

**Fabrication of human myobundles and culture**

The seeding chamber (4.5mm×1.8mm ×2mm) was formed by PDMS (Dow Corning, Sylgard 184 Silicone Elastomer Kit, 10:1) molds made from Teflon template. Four Ecoflex films were bonded on a nylon frame (Fig. 1a and Fig. S1) and the other end of the Ecoflex films was linked with a small piece of nylon (2.5mm×3.5mm). Then this Ecoflex bonding frame was placed into a PDMS mold previously treated with Pluronic F-127 (Sigma-Aldrich, P2443) and aligned with a seeding chamber in the mold. Myobundles were formed similar to our previously published methods [1]. HSkM cells were dissociated in 0.025% trypsin-EDTA (ThermoFisher, 25300062) to a single cell suspension and then encapsulated in the matrix gel. For each bundle $3 \times 10^5$ cells in 9.2 µl GM, 0.8 µl of 50 unit/ml thrombin (Sigma-Aldrich, T7513) in 0.1% BSA in PBS were mixed with 2 µl growth media, 4µl Matrigel, and 4µl of 20mg/ml fibrinogen (Sigma-Aldrich, F8630). All these steps were performed on ice. Then the mixed gel was pipetted into the seeding chamber. After gelation at 37 C for 30 minutes, the myobundles were incubated in GM containing 1.5 mg/ml 6-aminocaproic acid (ACA, Sigma-Aldrich, A7824). Myobundles were cultured in GM for 4–5 days to promote HSkM proliferation. Then, the frames with myobundles were removed from the molds. To promote fusion and differentiation, the media was replaced with shift media (SM), consisting of low glucose DMEM, 2% horse serum (Hyclone, SH30074.03), 10 µg/ml insulin (Sigma-Aldrich), 2 mg/ml ACA, and 1% antibiotic-antimycotic. and the myobundles were cultured dynamically on a rocker (0.33 Hz, Gene Mate) for the duration of the experiment. Contractile forces were obtained daily for the myobundles at room temperature. To maintain a constant pH in the media during force measurements, HEPES (4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid) buffer (Sigma-Aldrich, H0887) was added in the shift media. The culture media was changed immediately after forces were measured and the myobundles returned to the incubator.

Cerivastatin sodium salt hydrate (Sigma-Aldrich, SML0005) was prepared at 1000× stock solutions in PBS and sterile-filtered for use. 1 mM DL-Mevalonolactone (Santa Cruz, CAS 674-
26-0) was prepared at 1,000× stock solutions in PBS. 10 μM coenzyme Q10 (Sigma-Aldrich, C9538) was prepared at 10,000× stock solutions in DMSO, in which DMSO was used as vehicle control.

**Force Testing**

We first characterized the mechanical properties of the Ecoflex membrane. For characterization of the Ecoflex film, one end of the Ecoflex film (12mm×3mm×0.5mm) was pinned in the test chamber of force transducer (WPI Instruments, Part #: SI-BAM-21LC). Then the film was stretched by a stepper motor (Thor Labs, Part# Z812B) in 0.05 mm steps for a total distance of 0.5 mm (10 steps). At each step, the passive force of Ecoflex film was measured and the displacement of microbeads in film was recorded on video through a stereomicroscope (AmScope-MA1000, 1.5× objective).

**Ecoflex bonding myobundles testing:** To compare myobundle force generation by the force transducer and Ecoflex membrane displacement, a larger myobundle (50 μl gel with $7.5 \times 10^5$ cells/bundle) was cultured on a smaller frame (19 × 8 mm outer dimensions, 15 × 6.2 mm inner dimensions) directly. An Ecoflex film was bonded at one end of the outside frame (as shown in S1b). Then this Ecoflex bonding myobundle was pinned on the force transducer and the force was measured during stimulation (Grass SD9). At the same time, the stretch of the Ecoflex membrane induced by myobundle contraction was recorded as video through the stereomicroscope (1.5× objective). For twitch testing, the stimulating condition was 1 pulse at 1 Hz, 40V/cm. For tetanus testing, the stimulating condition was 20Hz, 20 pulses, 40V/cm.

**In situ active force testing:** The frame with myobundles array was pinned into a PDMS coated 6-well plate (Figs. S.2 a, b) and placed under a microscope. To avoid rubbing between the Ecoflex film and well bottom, the frame was pinned onto two PDMS slabs that were about 2 mm higher than the well bottom. To electrically stimulate the muscles (20Hz, 20 pulses), a pair of electrodes connected to a stimulator was placed into the well. At the same time, the stretch of all Ecoflex films were recorded as video through stereomicroscope (1.1× objective).

**In situ passive force testing:** The length of Ecoflex films before stimulation were measured every day from day 8 to day 14, and we normalized the Ecoflex length and force to the values on day 8. Finally, the static length change (without stimulation) of Ecoflex films were acquired over time, which was resulted from passive contraction of myobundles and the passive force was calculated.
using strain vs. force relation.

**PIV analysis**

Particle image velocimetry (PIV) analysis was performed using an ImageJ plugin (ImageJ-PIV) to quantify Ecoflex displacement. Images were obtained before stretch and when the Ecoflex films were stretched maximally. These two images were opened in ImageJ and stacked into one figure. Then the image was changed into 8-bit and a region of interest was chosen. The PIV plugin was used to obtain the cross-correlation between two image sub-regions and the displacement of 400-500 particles (optical flow) for the image pairs.

**Relation between strain and force of Ecoflex**

In force testing, the Ecoflex film was stretched at 0.05 mm/step for 0.5 mm (10 steps) and the passive force of Ecoflex film was measured at each step. At the same time, the displacement of microbeads in film was recorded as video through stereomicroscope. For each step, one image was captured. By application of ImageJ-PIV, the primary length of Ecoflex film (defined as L1 as shown in Fig. 3c) and the longest displacement of Ecoflex in each stretch step (defined as L2 as shown in Fig. 3c, and the Ecoflex film bonded on the frame area was chosen as the testing region), the strain of Ecoflex was calculated.

**Immunofluorescence**

Myobundles were washed in PBS then fixed in 4% paraformaldehyde for 25 min at 4°C. Following fixation, samples were washed in wash buffer (WB, PBS+1.5% goat serum+1%BSA+0.2% Triton X) then blocked in block buffer (PBS+10% goat serum+5%BSA+0.1% Triton X) for 1 hr in room temperature. Cross-sections were obtained on a cryostat (Leica CM3050). The following primary antibodies were used for tissue characterization: sarcomeric α-actinin (Sigma, 1:150) and F-actin (Sigma, 1:150) and were visualized with fluorescently labeled secondary antibodies (1:200) (Life Technologies) and Hoechst (1:1000). Oil Red staining was performed by using standard protocols on myobundles fixed in 4% paraformaldehyde. Images were acquired using a Leica SP5 inverted confocal microscope.

**Length measurement and density of the nuclei count.**

All the length and width of cells or myobundles were measured in ImageJ software. Then the pixel unit was switched to micrometer according to the reference ruler. The density of the nuclei was analyzed in ImageJ software by using the particle analysis tool (8-bit image, contrast was set as max, the threshold was set as 160-255).
Statistics
Data were analyzed using Originlab and Graphpad Prism. Results are presented as mean ± S.E. Statistical significance was determined by unpaired t-test. p < 0.05 was considered statistically significant, for N=3-6 samples (*p < 0.05, ** p < 0.01, *** p < 0.001). Coefficient of variation was analyzed by using standard deviation/mean×100%.

Modeling the contractile behavior of human skeletal muscle myobundles in series with Ecoflex membranes.

Recently, Schroer et al.[2] showed that a modified Hill model described the dynamic response of engineered cardiac tissue constructs to pharmacological agents and changes in length. This establishes the usefulness of applying the Hill model to examine the response of engineered human skeletal or cardiac muscle myobundles. To examine the influence of the Ecoflex membrane on the measured force, we examined the effect of adding the Ecoflex, a linearly elastic material, in series with a Hill model (Fig. S5a) consisting of an active element in parallel with a viscous element and spring. These three units act in series with a spring.

The active element viscous elements, and spring kPE are all in parallel and act, as a unit, in series with the series elastic element with spring constant kSE. The forces on the series elastic and active/viscous elements are the same and equal the myobundle force, FM:

\[ F_M = F_A + \eta \frac{dx_2}{dt} + k_{PE}x_2 = k_{SE}x_1 \]

(1)

The total extension of muscle is (Fig. S5a):

\[ x = x_1 + x_2 \]

(2)

The corresponding change in the muscle length with time is:

\[ \frac{dx}{dt} = \frac{dx_1}{dt} + \frac{dx_2}{dt} \]

(3)

Equations 2 and 3 can be used to replace \( x_2 \) and \( \frac{dx_1}{dt} \) in equation 1:

\[ F_M = F_A + \eta \left( \frac{dx}{dt} - \frac{dx_1}{dt} \right) + k_{PE}(x - x_1) \]

(4)

Using the relation \( F_M = k_{SE}x_1 \), \( x_1 \) can be eliminated.
\[ F_M = F_A + \eta \left( \frac{dx}{dt} - \frac{1}{k_{SE}} \frac{dF_M}{dt} \right) + k_{PE} \left( x - \frac{F_M}{k_{SE}} \right) \tag{5} \]

Likewise, \( x_T = x + x_p \) is constant because the movement of the muscle unit and Ecoflex is isometric. For isometric contraction of the Ecoflex and the myobundle,

\[ \frac{dx_T}{dt} = 0 = \frac{dx}{dt} + \frac{dx_p}{dt} \tag{6} \]

Further

\[ \frac{dx}{dt} = -\frac{dx_p}{dt} = -\frac{1}{k_{eco}} \frac{dF_M}{dt} \tag{7} \]

Replacing \( x \) and its derivative:

\[ F_M = F_A - \eta \left( \frac{1}{k_{eco}} \frac{dF_M}{dt} + \frac{1}{k_{SE}} \frac{dF_M}{dt} \right) + k_{PE} \left( x_T - \frac{F_M}{k_{eco}} - \frac{F_M}{k_{SE}} \right) \tag{8} \]

Rearranging yields the following expression:

\[ \left( 1 + \frac{k_{PE}}{k_{SE}} + \frac{k_{PE}}{k_{eco}} \right) F_M + \left( \frac{\eta}{k_{SE}} + \frac{\eta}{k_{eco}} \right) \frac{dF_M}{dt} = F_A + k_{PE} x_T \tag{9} \]

In the absence of the Ecoflex and with isometric contraction of the muscle, \( \frac{dx}{dt} = 0 \), equation 5 becomes

\[ F_M = F_A - \eta \frac{dx}{dt} + k_{PE} \left( x - \frac{F_M}{k_{SE}} \right) \tag{10} \]

Using the relation \( F_M = k_{SE} x_1 \)

\[ F_M = F_A - \eta \frac{dF_M}{dt} + k_{PE} \left( x - \frac{F_M}{k_{SE}} \right) \tag{11} \]

Rearranging yields:

\[ \frac{dF_M}{dt} = \frac{k_{SE}}{\eta} \left( F_A + k_{PE} x - \left( 1 + \frac{k_{PE}}{k_{SE}} \right) F_M \right) \tag{12} \]

The values of parameters for the simulations were derived as follows. From Figure 3b, the slope of the force-stretch curve, 57.8±1.4 mN/cm, equals \( k_{eco} \). The passive force curve was measured under steady state and the overall spring constant is the sum of each spring constant.

\[ F = k_o \Delta x = (k_{PE} + k_{SE}) \Delta x \tag{13} \]
From a plot of the passive force versus length for same donor used in Figure 2, the spring constant, \( k_{PE} + k_{SE} \), is 8.3±0.3 mN/cm.

To model a single twitch, we used the following function to describe the force generated by the active element:

\[
F_A = 250 t e x p\left( -15t^{0.5} \right) 
\]

This function yielded a twitch force peak force comparable to what is measured with myobundles (~0.3 mN) and was of duration similar to replicate twitch of myofibers [3].

To estimate the value for \( \eta \), we first examined the decline in force following a twitch stimulus as shown in Figure 2b. The force is approximately 0.01 mN by 0.14 s. Solving equation 12 following the ending of the twitch stimulus (\( F_A = 0 \)), the decline in the active force with time proceeds as \( F_R e x p\left( -(k_{SE}+k_{PE})(t-t_R)/\eta \right) \) where \( F_R \) is the force at \( t_R \). This reference time was slightly greater than 0.14 s. For the twitch curves shown, a plot of the natural logarithm of the force after the peak was linear in time (\( r^2 > 0.98 \)) and equal to -11.2±2.0 s\(^{-1}\), which equals \( -(k_{SE}+k_{PE})/\eta \). Thus, \( \eta = (k_{SE}+k_{PE})/11.20 \approx 8.3/11.20 = 0.74±0.13 \) mN s/cm. Analysis of the decline in active tetanus force after end of stimulation shown in the same figure yields a similar estimate of \( \eta = 0.80±0.01 \) mN s/cm.

Using a MATLAB nonlinear regression routine, nlgreyest, we found that a broad range of parameter values fit the twitch results. By adjusting the values of \( \eta, k_{PE}, \) and \( k_{SE}, \) the best fit values were \( \eta = 0.70 \) mN s/cm, \( k_{PE} = 1 \) mN/cm and \( k_{SE} =10.6 \) mN/cm. The experimental results and simulation agreed well (Figure S5b).

Using these parameter values, MATLAB programs were written to simulate the twitch and tetanus response. Simulation results for the addition of the Ecoflex membrane in series with the myobundle causes a decrease in the maximum twitch force and tetanus force, similar to that that observed in Figure 2 (Figure S6). For tetanus, the ratio of the forces with the Ecoflex to without the Ecoflex are 90%.
Literature Cited


Supplementary Figures and Legends

Supplementary Figure S1: a. Schematic diagrams depicting the process for fabricating myobundles. b. Myobundle attachment to Ecoflex membrane for comparison of force using force transducer and Ecoflex.
Supplementary Figure S1: a. Schematic diagrams depicting the process for fabricating myobundles. b. Myobundle attachment to Ecoflex membrane for comparison of force using force transducer and Ecoflex.

Supplementary Figure S2: a. Schematic figure of in situ Ecoflex monitoring system. b, Actual image of in situ monitoring system: stimulator, stereoscope, electrode and 6 well plate.
Supplementary Figure S3: Characterizations of Ecoflex. (a) the actual imaging of Ecoflex film, (b) the distribution of microbeads in Ecoflex. (c) the stability of Ecoflex during long term exposure with media. (d) the stability of Ecoflex among different batches. (n=3, data presented as mean ±S.D.)
Supplementary Figure S4: The characterization of four myobundles in a frame. a. The bright field image of four myobundles in a frame. b. The largest displacement of Ecoflex films during myobundles contraction in tetanus (ImageJ-PIV analysis results, 1 pixel = 10.7 µm).
Supplementary Figure S5.  a. Schematic of Hill model of engineered human skeletal muscle myobundles. The model consists of an active source of skeletal muscle contraction A, with force $F_A$ arranged in parallel with a viscous element with constant $\eta$ (dyne s cm$^{-1}$) and a spring defined by a constant $k_{PE}$ (dyne/cm). This contractile unit is in series with a spring defined by a constant $k_{SE}$ (dyne/cm). The muscle unit acts in series with the Ecoflex membrane which has a spring constant $k_{eco}$ (dyne/cm). b. Comparison of model simulation and measured value for twitch force of myobundles attached to frame. Parameter values were: $\eta = 0.4$ dyne s/cm, $k_{PE} = 0.8$mN/cm, $k_{SE} = 7.5$ mN/cm, $k_{eco} = 57.8$ mN/cm and $x_T = 1.4$ cm. The passive force was subtracted from the results to only show active force for contraction.
Supplementary Figure S6. Simulation of twitch (a) and tetanus (b) using the Hill model and parameters listed in the legend to Figure S5.
Supplementary Figure S7: a. Timeline for assessment of drug treatment on myobundles. b. Normalized passive force following treatment with 100 nM cerivastatin with 0, 0.1, 0.3 or 1 mM mevalonate. c. The normalized specific force (active force/cross-sectional area) following treatment with 100 nM cerivastatin with 0, 0.1, 0.3 or 1 mM mevalonate. d. Relative passive force following treatment with 0, 0.1, 0.3 or 1 mM mevalonate. e. Coefficient of variation in tetanus force measured daily for conditions in Figure 5. All force or width were revised/normalized to day 8 (n=4-6, data presented as mean ±S.E.), * p < 0.05; ** p<0.01 relative to control conditions.
Supplementary Figure S8: Passive force (a) and the diameter change (b) of myobundles under treatments of cerivastatin and CoQ10 for a week. All force or width were revised/normalized to day 8 (n=4-6, data presented as mean ±S.E.), * p < 0.5; ** p<0.01 relative to control conditions.

References

Supplementary Video 1: Contraction of myobundles independently and synchronously
Supplementary Video 2: Spontaneous beating of myobundles