Supporting Information

Interface-governed electromechanical coupling in bioinspired hierarchical piezoelectric poly(L-lactide) architectures

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Experimental

Materials

Poly-L-lactic acid (RESOMER L 207 S, Evonik Operations GmbH), calcium nitrate pentahydrate (Sigma-Aldrich, Germany), ammonium dihydrogen phosphate (Sigma-Aldrich, Germany), urea (Alfa Aesar, Germany), ultrapure water (Purelab Option Q7, ELGA), chloroform (Sigma-Aldrich, Germany), dimethylformamide (DMF, Sigma-Aldrich, Germany), Dulbecco's modified Eagle's medium-high glucose (DMEM, Sigma-Aldrich), fetal bovine serum (FBS, Gibco, US), penicillin–streptomycin antibiotics (PS, 1:1, Gibco, USA), Dulbecco's phosphate buffered saline (DPBS, Sigma-Aldrich, USA), TrypLE select (Gibco, USA), poly-L-lysine solution (Bio Reagent, Sigma, UK), Presto Blue Cell Viability Reagent (Molecular Probes, Invitrogen, Thermo-Fisher Scientific), paraformaldehyde (Sigma-Aldrich, Germany), Triton X 100 (Sigma-Aldrich Life science, USA), Rhodamine Phalloidin (RP, Invitrogen by Thermo Fisher Scientific, USA), and DAPI (diamidino-2-phenylindole, Biotium, Fremont, CA), Hexamethyldisilazane (HMDS, Sigma-Aldrich, Germay) were used.

Methods

Processing and synthesis filler particles

Hydroxyapatite (HAp) filler was synthesized using a modified sonochemical method, with urea employed as the precipitation agent.[24] Aqueous solutions of calcium nitrate pentahydrate and ammonium dihydrogen phosphate were mixed and heated to boiling. Urea was then added, and the reaction mixture was maintained at 90 °C for 4.5 h. After heating was discontinued, stirring was continued for an additional 24 h at room temperature. The resulting precipitate was collected by centrifugation, washed with water, and stored in ultrapure water under refrigeration.

Carboxymethyl-modified bacterial nanocellulose (BC_{CMC}) fillers were prepared using commercial bacterial nanocellulose (BC; fzmb GmbH, Germany) and sodium carboxymethyl cellulose (CMC, 250 kDa; Sigma-Aldrich), following our previously reported protocol[25] with modifications to the CMC addition and acid hydrolysis steps. Briefly, an autoclaved wet BC membrane (9 g) was cut into small pieces, disintegrated, and dispersed in 70 mL of a 0.1 wt% aqueous CMC solution by homogenization at 15000 rpm for 3 min, followed by 20000 rpm for an additional 15 min. The final dispersion had a concentration of 2.4 mg mL^{-1}.

Processing drawn films. Five-times drawn (DR5) PLLA films containing 1 wt% HAp filler (denoted as PLLA HAp DR5) were prepared by solvent casting followed by hot pressing and uniaxial drawing, as described previously.[13] PLLA was dissolved in chloroform (10 wt/v%) under magnetic stirring until complete dissolution, after which HAp dispersed in DMF (1 wt% relative to the polymer) was added. The mixture was stirred until homogeneous, poured into a Petri dish, and left to evaporate. Non-piezoelectric, non-drawn (DR1) films were obtained by hot pressing the resulting solvent-cast material in an aluminium foil mould at 250 °C (Kambič, Slovenia) for 10 min using a 50 kN manual press (Weber, Germany), followed by rapid cooling in a water–ethanol mixture. Piezoelectric DR5 films were subsequently produced by heating the DR1 films to 80 °C and stretching them to five times their original length, according to a previously established procedure.[14]

Processing of electrospun fibres. Networks of PLLA fibres containing BCCMC (denoted as PLLA BC_{CMC} fibres) were fabricated by electrospinning. PLLA was dissolved in chloroform at a concentration of 9 wt/v%, and BC_{CMC} previously dispersed in DMF (2 mg BC_{CMC} in 2 mL DMF; 0.22 wt% relative to the polymer) was added. Electrospinning was carried out using a rotating-drum electrospinning system (Linari Engineering S.r.l., Italy) at an applied voltage of 29 kV, a needle-to-collector distance of 10 cm, and a drum rotation speed of 700 rpm, under ambient temperature and humidity.

Forming drawn film with fibres on the surface. Films with joined morphologies were prepared using two different approaches. In the first approach, PLLA BC_{CMC} fibres were deposited onto an already drawn PLLA

HAp DR5 film, yielding PLLA HAp DR5/BC_{CMC} fibres. Prior to bonding, the surfaces of the PLLA HAp DR5 film and the PLLA BC_{CMC} fibres intended for contact were treated with atmospheric-pressure argon plasma using a plasma jet (kINPen IND-Lab, Germany). The activated surfaces were then brought into contact and pressed together at room temperature using a manual press (50 kN).
In the second approach, PLLA BC_{CMC} fibres were deposited onto a non-drawn PLLA HAp DR1 film, after which both layers were drawn together to form PLLA HAp DR5/BC_{CMC} DR5 fibres. For this process, the PLLA BC_{CMC} fibres and PLLA HAp DR1 film were plasma-treated and pressed together following the same procedure as described above. The bonded bilayers were subsequently annealed at 80 °C and stretched to five times their original length, resulting in uniaxially oriented films and fibres.

Characterization. X-ray diffraction (XRD) of film and fibre samples was performed using an Empyrean diffractometer with a PIXcel3D 1×1 detector (2θ = 5–60°, step size 0.026°). Small- and wide-angle X-ray scattering (SAXS/WAXS) measurements were carried out on a SAXS Point 2.0 system (Anton Paar) at the BIOCEV Centre, equipped with a gallium source (λ = 1.34 Å) and an Eiger R 1M detector. Data were collected at room temperature in transmission mode after calibration with silver behenate, using sample-to-detector distances of 572 and 79 mm. Differential scanning calorimetry (DSC; NETZSCH STA 449 C/6/G Jupiter) was used to determine crystallinity by heating samples from 35 to 220 °C at 20 °C min^{-1}. The degree of crystallinity was calculated from the melting and cold-crystallization enthalpies using $\Delta H_{100}\% = 93.6\ J\ g^{-1}$ for PLLA.[14] Molecular orientation was analysed by polarized Raman spectroscopy (NTEGRA Spectra, NT-MDT) using a 488 nm laser (200–3100 cm^{-1}). Orientation was calculated from the HHH/HVV intensity ratio at 875 cm^{-1} and normalized to the C=O band at 1772 cm^{-1}, as described earlier.[16] Surface morphology was examined by SEM (JSM-7600F, JEOL) at 10 kV after gold sputtering. Surface chemistry and wettability were assessed by methylene blue staining (0.1 wt%) and water contact angle measurements (Theta Lite, Biolin Scientific; ~3 µL droplets).

Adhesion forces in two-layered structures. The adhesion forces were measured with a custom-built extensometer designed for thermomechanical measurements of soft materials. The device consists of a heating chamber in which the film-formed sample is clamped between two holders, with the bottom stationary holder attached to a strain gauge and the upper holder to a translation stage. In our case, the examined film was fixed in the bottom holder, while the peeled-off fibers were clamped in the upper holder. During peeling, only the upper holder moved, while the applied force was measured on the bottom holder. Peeling was performed at ambient temperature, with a constant velocity (~12.5 µm/s) and at a 0° angle, i.e., in the direction parallel to the film. The average peeling force, calculated as $P = F/w$ where F is the measured force and w is the sample width, was determined by averaging the values from the first observed maximum to the end of the measurement.

Piezoelectric measurements. Piezoelectric activation was performed using US stimulation at frequencies of 80 kHz and 1 MHz to induce mechanical deformation. Low-frequency stimulation was applied using an ultrasound bath operating at 80 kHz and 100% intensity, while high-frequency stimulation was carried out at 1 MHz with a power density of 3 W cm^{-2}, following a previously reported protocol.[13] Copper electrodes were attached to the edges of the samples along the drawing direction and insulated with Kapton tape to prevent water ingress. During US stimulation, the electrodes were exposed to ultrasound, and the generated electrical signals were recorded using a digital oscilloscope (Keysight MSOX3034T).

Plasma treatment of materials before cell testing. Prior to cell-interaction studies, the materials were additionally treated with atmospheric-pressure argon plasma. Samples (10 × 10 mm^2) were prepared, and the surface intended for cell contact was plasma-treated for 60 s at a gas flow rate of 1.7 L min^{-1} by scanning the plasma jet over the sample at a distance of 10 mm.

Adhesion and cytotoxicity of human keratinocytes (HaCaT). HaCaT cells (TCC PCS-200-011) at low passages (≤4) were cultured in complete growth medium (DMEM supplemented with 10% FBS and 1% penicillin–streptomycin) and seeded directly onto the surfaces of the tested films (10 × 10 mm^2). Prior to seeding, the films were immersed in poly-L-lysine solution (300 µL) and sterilized by incubation in complete medium for 2 h. Cells were seeded at a density of 20000 cells per well in 24-well plates and incubated for 24 h at 37 °C

and 5% CO_2 (MCO-19AIC(UV)-PE incubator, Panasonic, Japan). Cell viability was assessed by fluorescence measurement (Ex/Em = 560/590 nm; Synergy H1 plate reader, Biotek) following the addition of 10 wt% Presto Blue Cell Viability Reagent and 1 h incubation. To quantify cell adhesion, the films with adhered cells were washed with DPBS, transferred to new 24-well plates, and the fluorescence measurement was repeated. Experiments were performed in triplicate and repeated at least twice.

Piezostimulation of HaCaT cells. To assess piezostimulation, films with adhered cells were washed, refreshed with complete DMEM, and exposed to ultrasound at 80 kHz (30% power, 20 min) or 1 MHz (1.8 W cm^{-2}, 1:10 duty cycle, 3 min), followed by 24 h incubation. Non-stimulated samples served as controls. Stimulation was repeated daily for three days, with cell viability measured after each cycle. Experiments were performed in triplicate and repeated at least twice.

Actin filaments. After the three-day stimulation period, actin filament formation was examined. Cells were washed with DPBS, fixed in 3.7% paraformaldehyde in DPBS, permeabilized with 0.5% Triton X-100 in DPBS, and washed again. The cells were then stained with rhodamine–phalloidin (1 µL mL^{-1}) for actin filaments and DAPI (5 µL mL^{-1}) as a nuclear counterstain, and imaged using a fluorescence microscope (Eclipse Ti-U inverted microscope, Nikon). Fluorescence intensities were also quantified using a plate reader (rhodamine–phalloidin: Ex/Em = 584/562 nm; DAPI: Ex/Em = 355/346 nm). Experiments were performed in triplicate and repeated at least twice.

Cell morphology. After the proliferation experiments, HaCaT cells on the materials were fixed with 2.5% glutaraldehyde in DPBS, washed with DPBS, and dehydrated through a graded ethanol series (10–100%), followed by treatment with 50% hexamethyldisilazane (HMDS) in ethanol and 100% HMDS. Samples were then air-dried, sputter-coated with gold, and examined by SEM to assess cell morphology.

Statistical analysis. Data are presented as mean ± standard deviation (SD). Graphs and statistical analyses were performed using GraphPad Prism software. Statistical significance was evaluated by one-way ANOVA, with *, **, ***, and **** indicating $p < 0.05$, $p < 0.005$, $p < 0.001$, and $p < 0.0001$, respectively.

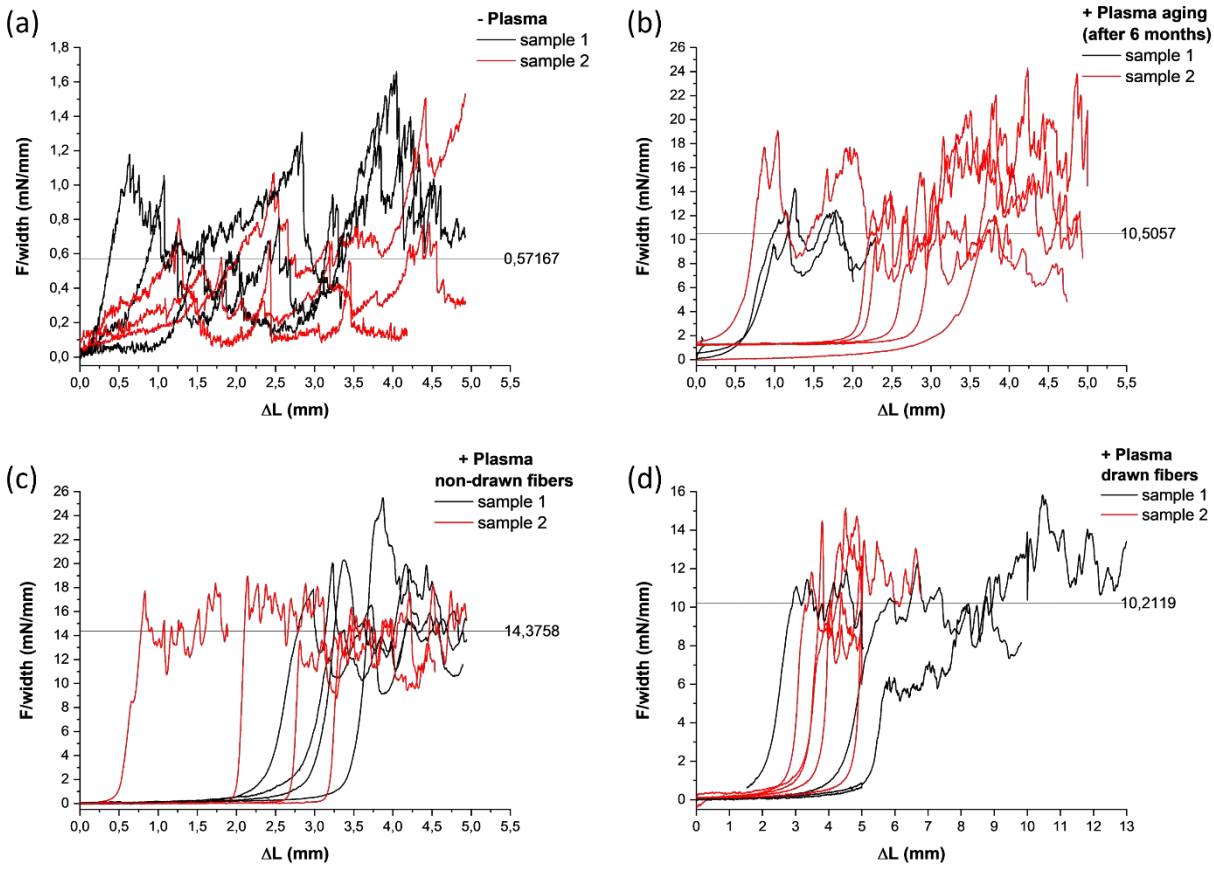
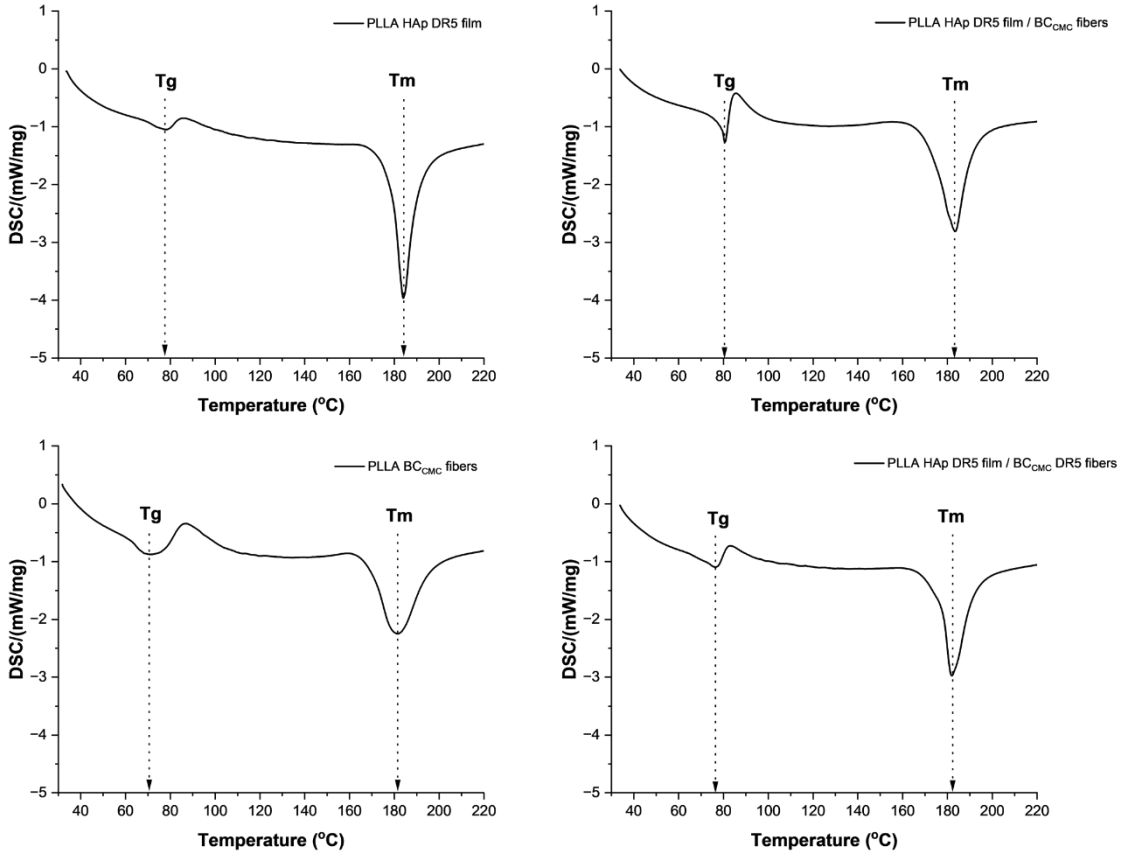


Figure S1. Superimposed peeling measurements, the distance between the holders with respect to the peeling force, for the two-layered materials: no plasma (a), plasma aging (b), non-drawn top fibers (c), and drawn top fibers (d).



Material	Tg(°C)	Tm(°C)
PLLA Hap DR5 film	76	182
PLLA BC$_{CMC}$ fibers	71	182
PLLA Hap DR5 film / BC$_{CMC}$ fibers	80	183
PLLA Hap DR5 film / BC$_{CMC}$ DR5 fibers	76	182

Figure S2. DSC patterns of PLLA materials and glass transition (Tg) and melting (Tm) temperature values**.**

Table S1. Calculated peak-to-peak piezoelectric values from the voltage generated on the materials surface while US deformed.

Material	Peak-to-peak values (mV) @ 80kHz US deformation	Peak-to-peak values (mV) @ 1MHz US deformation
PLLA Hap DR5 film	8,716	273,540
PLLA BC_{CMC} fibers	8,425	191,808
PLLA Hap DR5 film / BC_{CMC} fibers	22,903	407,213
PLLA Hap DR5 film / BC_{CMC} DR5 fibers	20,780	301,690

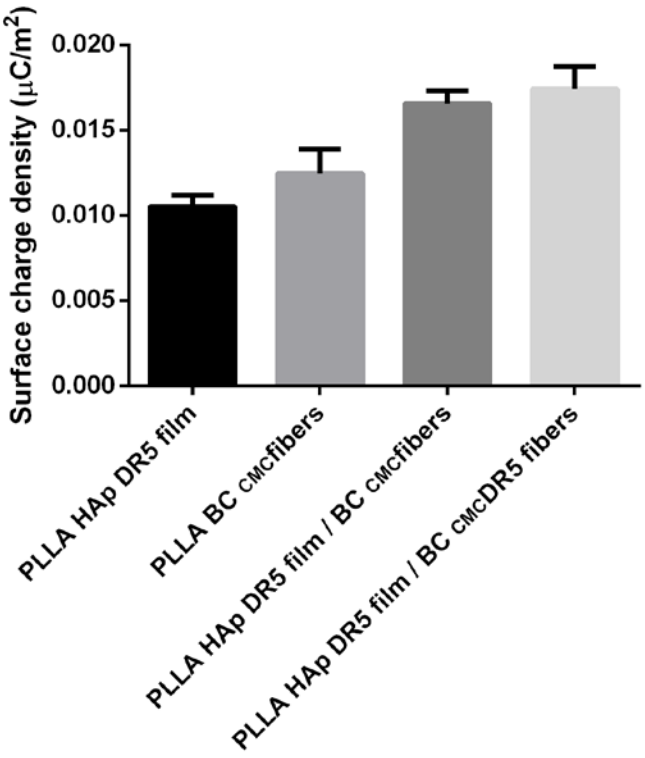


Figure S3. Charge density detected on single- and bilayer structures after mechanical deformation with 80 kHz ultrasound.

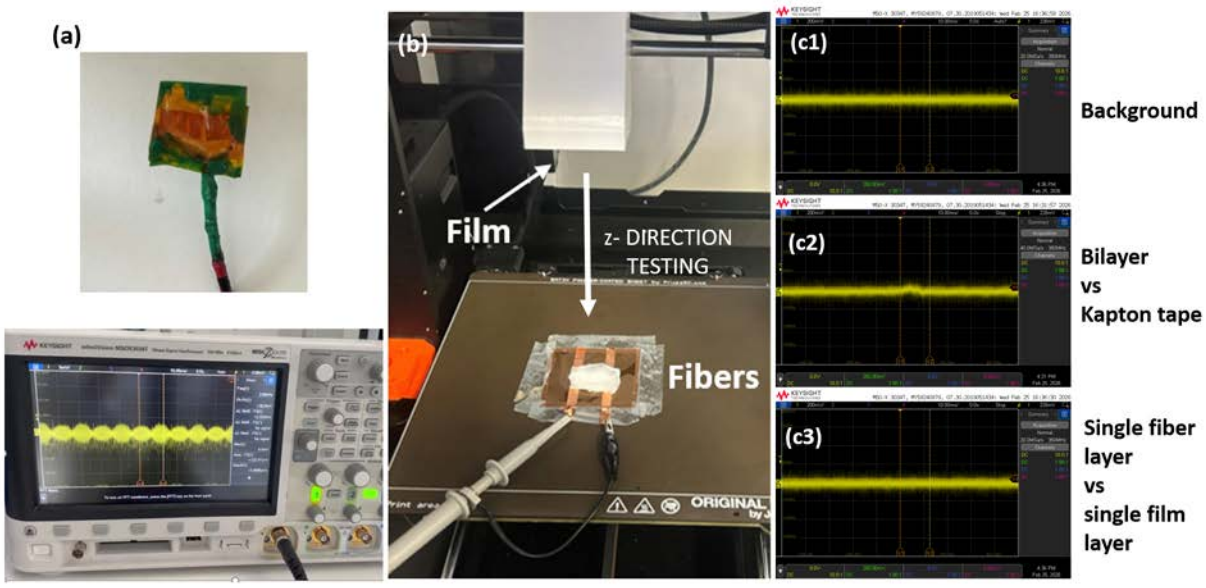


Figure S4. Control triboelectric testing: the set up for piezoelectric measurements – sample with electrode and oscilloscope –detected signal (a), set up for triboelectric measurements – z direction testing (b), the signals for tribo-testing- background (c1), friction between bilayer and Kapton type (c2) and friction between single fiber layer and single fiber layer (c3).

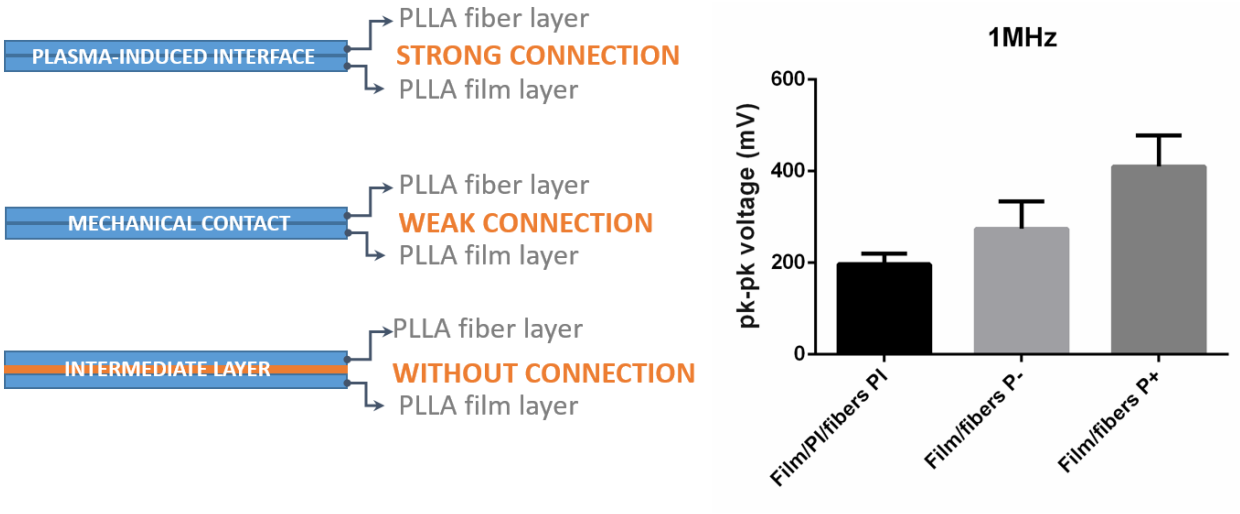


Figure S5. Functional comparison of two bilayered structures: illustration of the system designs with different levels of interface strains, including - strong interfacial strain (film and fiber layers connected by pressing after plasma treatment) (a), weak interfacial strain (film and fiber layers mechanically pressed without any plasma-pretreatment) and without interfacial strain (film and fiber layer were separated by intermediate layer), and measured voltage output of the three systems after mechanical deformation using 1MHz US.

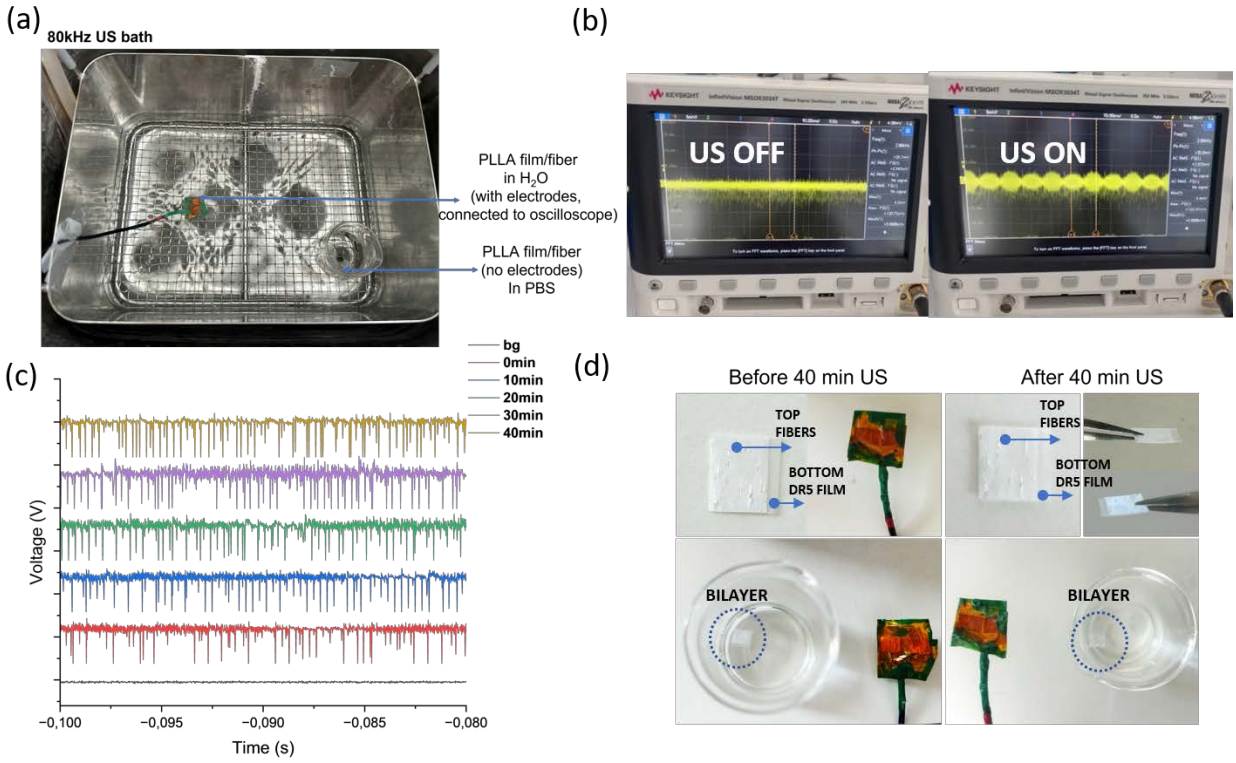


Figure S6. Materials stability testing: set up including PLLA film/fiber films immersed in liquid media- the sample with electrodes connected to oscilloscope (for functional stability test) and the sample without electrode directly inside a glass with PBS (for mechanical stability test) (a); the functional stability of the bilayer structure- voltage output measured for background (US OFF) and with mechanical stimulation (US ON) (b); the voltage signal detected after 0, 10, 20, 30 and 40 min US stimulation (c), and macroscopic view of the bilayer (with and after electrodes) before and after 40-minutes long US stimulation (d).

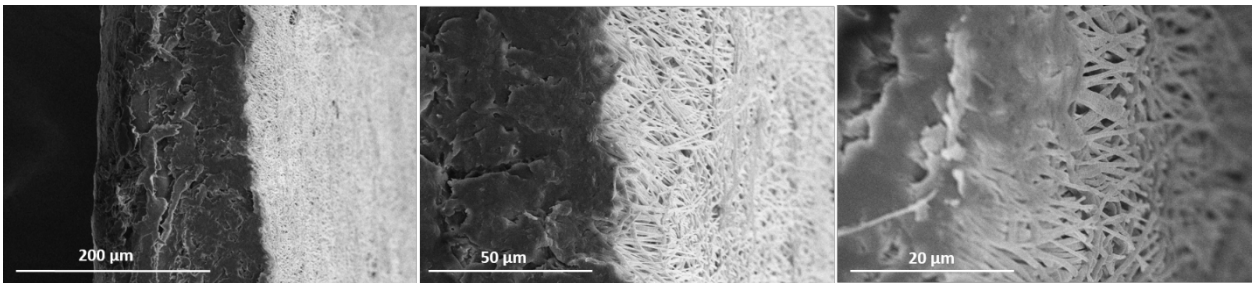


Figure S7. Cross-section of the bilayer structure formed of PLLA HAp film and PLLABC$_{CMC}$ fibers showing high level of morphological integration obtained using plasma pre-treatment.

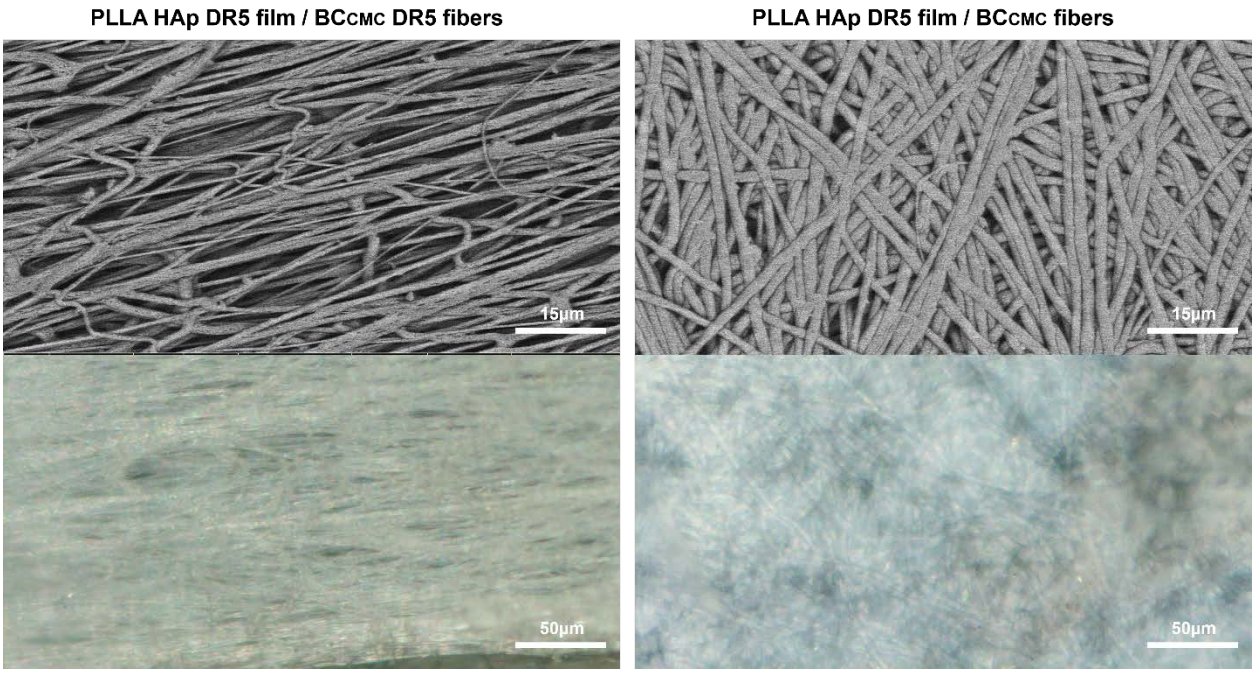


Figure S8. Fibers orientation in film/fibers materials observed under scanning electron microscope and optical microscope.

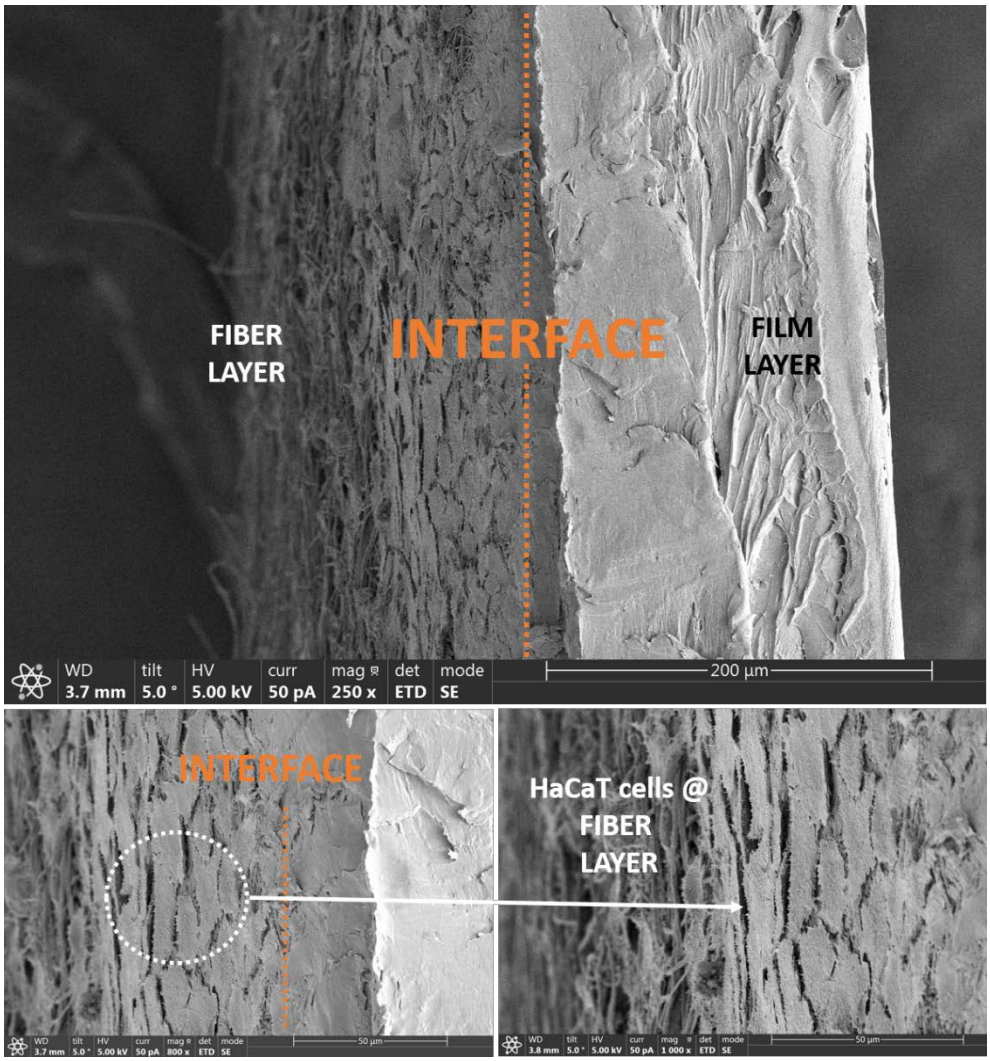


Figure S9. SEM images showing compactness of the bilayer interface after 5 day-incubation in biological growth medium and daily periodical stimulation with US followed by attaching and growing human HaCaT cells on top fiber layer.

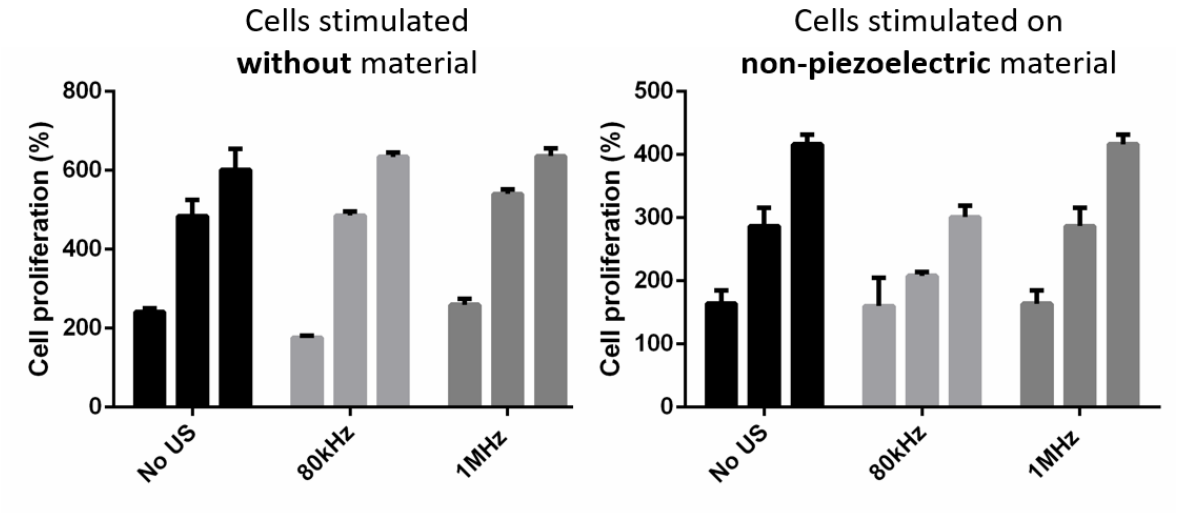


Figure S10. Proliferation of HaCaT cells grown without material substrate (directly on tissue culture plate) and using non-piezoelectric substrate (PLLA DR1) surface, following stimulation without or with 80-kHz and 1-MHz US.

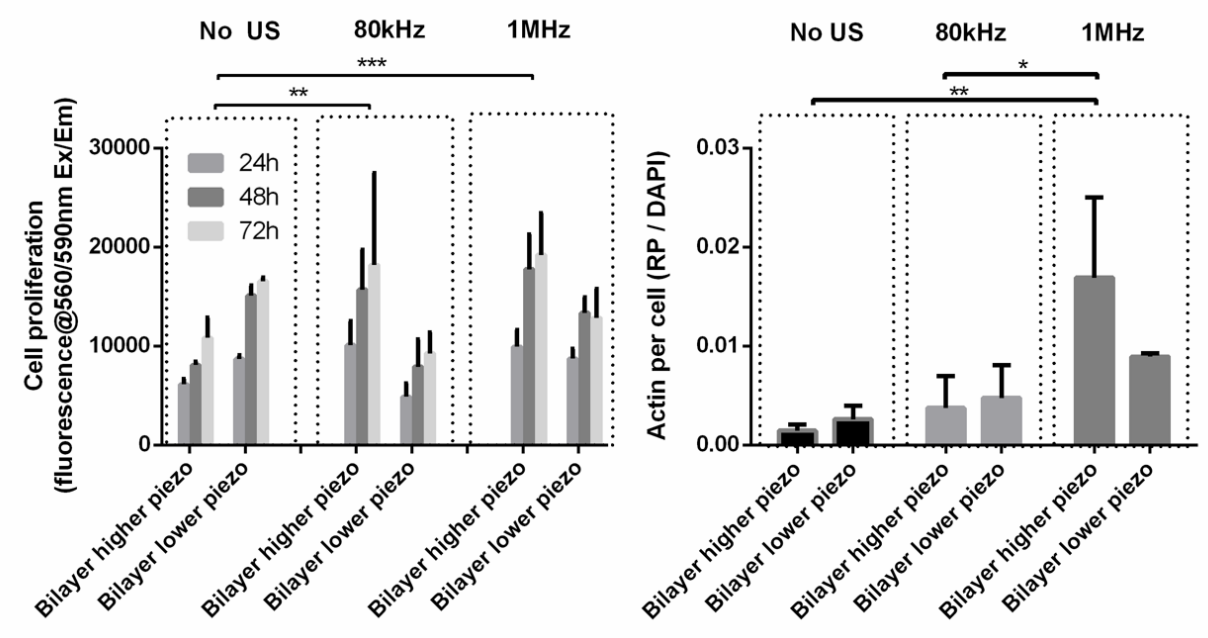


Figure S11. Comparison of cellular responses on low- and high- piezo bilayers: including proliferation and actin filaments formation after stimulation using 80 and 1MHz US taking PLLA Hap DR5 film/ PLLA CNC fibers as high piezo and PLLA Hap DR5 film/ PLLA CNC fibers DR5 as low piezo bilayer

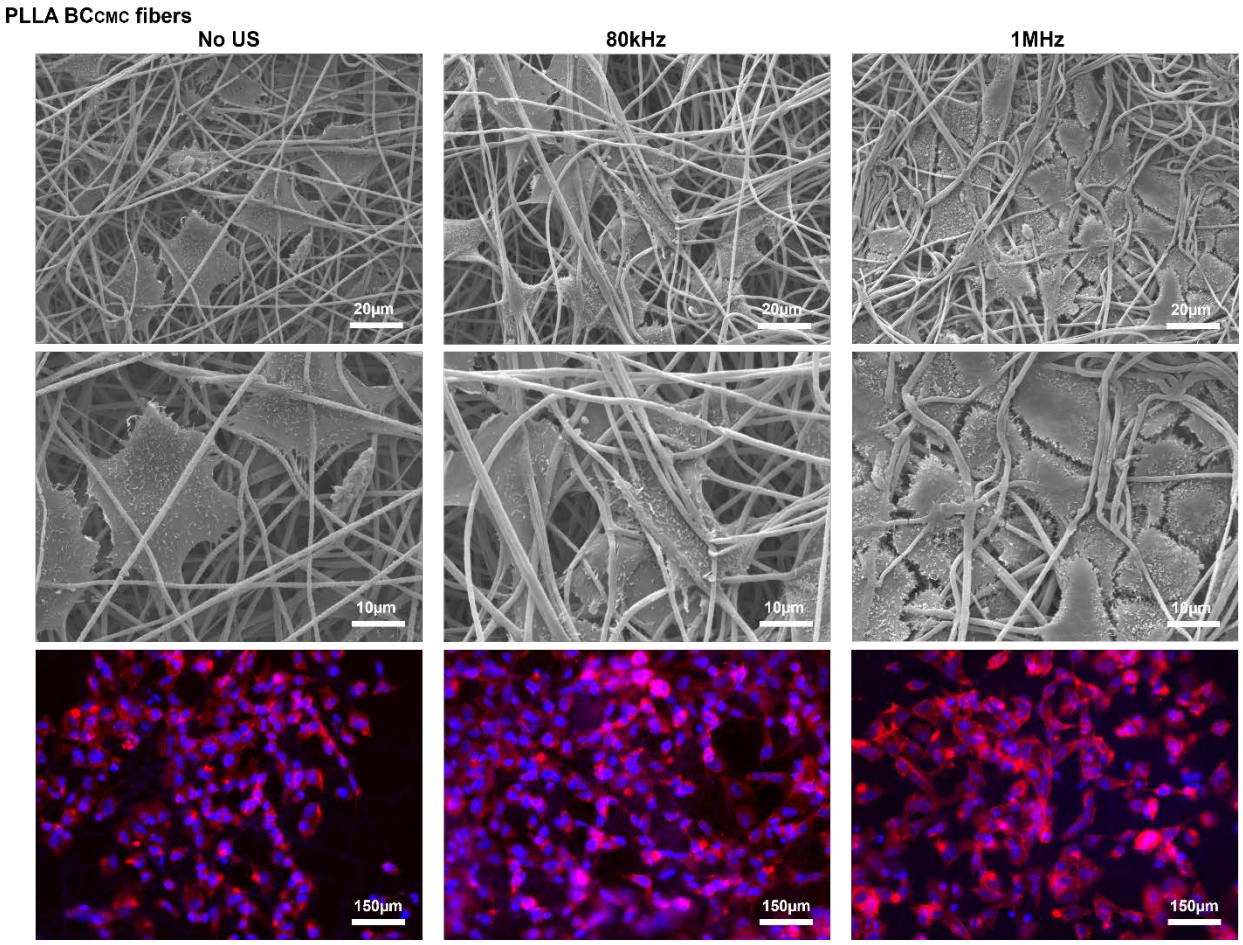


Figure S12. HaCaT cells morphology and actin filaments on the surface of PLLA BC_{CMC} fibers without (No US) and with (80kHz and 1MHz) ultrasound stimulation.

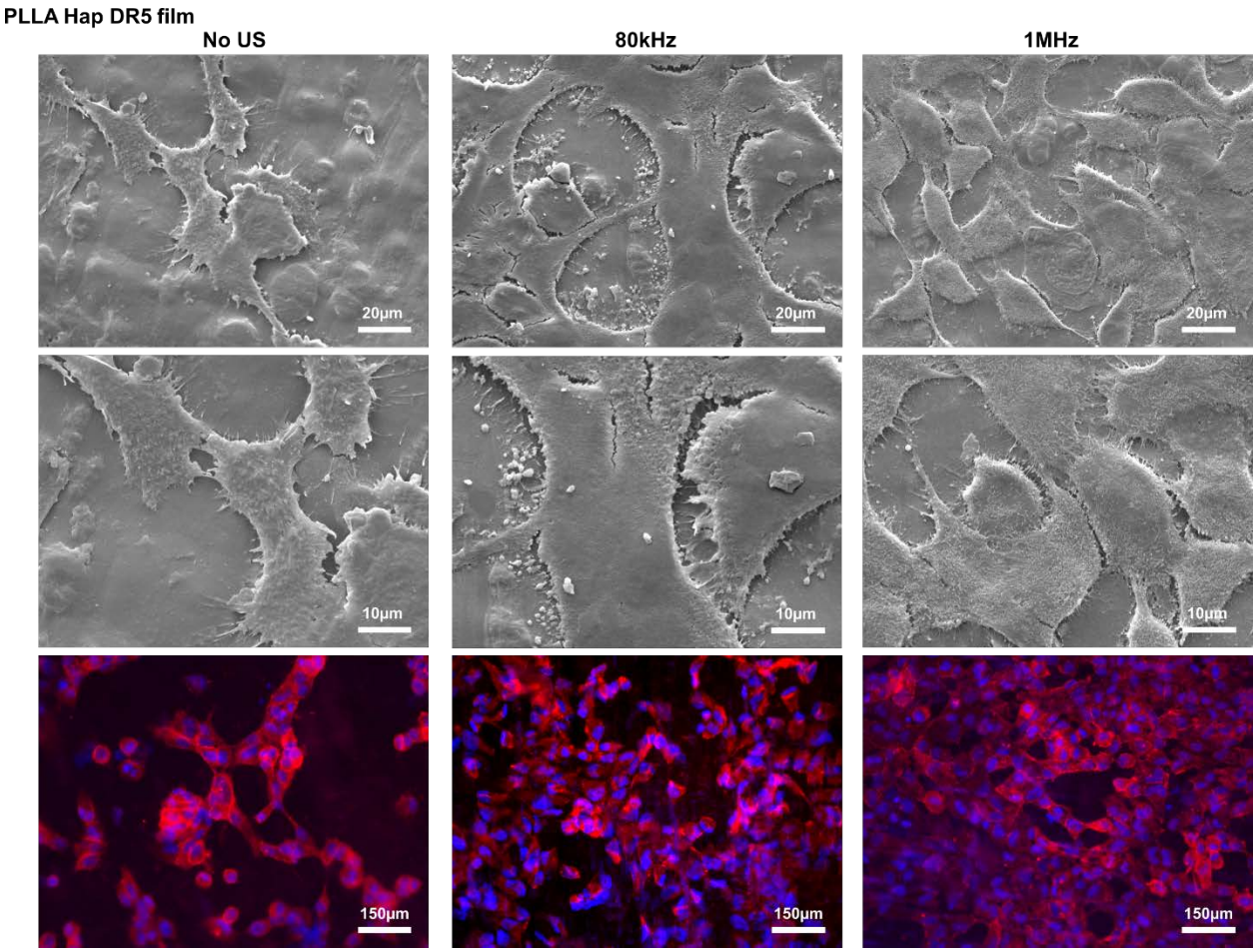


Figure S13. HaCaT cells morphology and actin filaments on the surface of PLLA HAp DR5 film without (No US) and with (80kHz and 1MHz) ultrasound stimulation**.**