Filler-Enhanced Piezoelectricity of Poly-L-Lactide and Its Use as a Functional Ultrasound-Activated Biomaterial
Filler-Enhanced Piezoelectricity of Poly-L-Lactide and Its Use as a Functional Ultrasound-Activated Biomaterial

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Poly-L-lactide (PLLA) offers a unique possibility for processing into biocompatible, biodegradable, and implantable piezoelectric structures. With such properties, PLLA has potential to be used as an advanced tool for mimicking biophysical processes that naturally occur during the self-repair of wounds and damaged tissues, including electrostimulated regeneration. The piezoelectricity of PLLA strongly depends on the possibility of controlling its crystallinity and molecular orientation. Here, it is shown that modifying PLLA with a small amount (1 wt%) of crystalline filler particles with a high aspect ratio, which act as nucleating agents during drawing-induced crystallization, promotes the formation of highly crystalline and oriented PLLA structures. This increases their piezoelectricity, and the filler-modified PLLA films provide a 20-fold larger voltage output than nonmodified PLLA during ultrasound (US)-assisted activation. With 99% PLLA content, the ability of the films to produce reactive oxygen species (ROS) and increase the local temperature during interactions with US is shown to be very low. US-assisted piezostimulation of adherent cells directly attach to their surface (such as skin keratinocytes), stimulate cytoskeleton formation, and as a result cells elongate and orient themselves in a specific direction that align with the direction of PLLA film drawing and PLLA dipole orientation.

1. Introduction

If injured, salamanders simply grow new body parts (including new limbs, heart, or parts of central neural system) and repair their functions. In contrast to mammals, salamanders have a natural capacity to change electrical potential at the wound site as a major alarm signal for cells to start regeneration. Regeneration in humans is not this advanced. However, there is much well-documented evidence showing that optimized, external electrical signals can target essential processes that direct cellular growth, stimulate tissue formation and lead to regeneration. Mimicking biophysical processes that naturally occur during self-repair of wounds and damaged tissues inside a living organism offers many possibilities for designing advanced regeneration strategies based on electrostimulation. In addition to stimulation of electrically excitable tissues (such as neural and muscle), known for their response to electrical clues, particular challenges are nonelectrically excitable tissues (including skin and bone), which also have voltage-sensitive channels and may respond to electrostimulation as well. However, new solutions in biomaterial design are needed.

Ultrasound (US)-activated biomaterials open a particularly interesting option for noninvasive, on-demand electrostimulated regeneration inside an organism without any use of electrodes. Organic piezoelectrics, which are able to polarize and generate surface charge upon mechanical deformation...
contribution to the piezoelectric properties of PLLA. A very similar effect was observed when hydroxyapatite (HAp), as a nonelectroactive filler, was used. At high concentrations (5–30 wt%), it was able to affect the crystallization of PLLA,[29] consequently influencing its piezoelectric response. Since hexagonal HAp does not exhibit piezoelectricity, its contribution was assigned solely to its effect as a filler on the piezoelectricity of PLLA. A very similar effect exists in bone, where its mineral part (which is mainly HAp) supports the structural organization of collagen and prevents excessive water adsorption and hydrogen bonding, which affects the fibril structure and diminishes the piezoelectricity of bone.[36–39] Although the impact of BT and HAp fillers on the crystallization of PLLA is well studied, many questions about their contribution to the polymer chain orientation and the consequent influence of this orientation on the piezoelectricity remain open.

Taking advantage of previous studies, the aim of this work was to clarify the contribution of piezoelectric and nonpiezoelectric fillers to the piezoelectricity of PLLA, especially in relation to their effect on the crystallinity and chain orientation. We particularly focused on PLLA modified with small contents of fillers and drew correlations of their properties with the filler morphology and anisotropy. To the best of our knowledge, the US activation of filler-modified PLLA films was explored for the first time (particularly regarding their capacity to form reactive oxygen species (ROS) and increase the local temperature), which was also applied in a US-mediated piezostimulation of human skin cells as a nonelectrically excitable in vitro model.

2. Results

Designing PLLA as an ultrasound-activated biomaterial followed the idea of using a filler-modification approach, see Figure 1. The approach considers adding a low content of fillers into the PLLA matrix, which was expected to influence polymer chain orientation and crystallinity, consequently enhancing its piezoelectricity and enabling ultrasound-activated biomaterials. PLLA was modified with HAp nanorods (HAp NRs) as a nonpiezoelectric filler and a series of BT (nano)structures with various morphologies and different degrees of tetragonality, including nanospheres (BT NPs), nanosheets (BT NSs), microblocks (BT MBs), and nanotextured rods (BT NTRs), as piezoelectric fillers characterized by different piezoelectricity.

2.1. Filler-Modified PLLA Films—Effect of Fillers on Polymer Orientation

Morphologically and structurally, BT filler particles are characterized by different aspect ratios (length/width/thickness) and high crystallinity (Figure 2a). The lowest aspect ratio was typical for BT NPs with a spherical-like shape (~300 nm diameter) (Figure 2a1,3) and high crystalline nature (Figure 2a5; and Figure S1, Supporting Information). These shapes were followed by BT NSs and BT MBs with higher aspect ratios, as they had a 2D morphology with thicknesses ranging from 100 to 200 nm for BT NSs and from 300 to 500 nm for BT MBs and micrometer-sized lengths (Figure 2a4,5,a10,11). The mosaicity of
the BT NSs (Figure 2a 4,5) implied their mesocrystalline structures, which were also confirmed by the single crystalline SAED pattern (Figure 2a 6), indicating <100> orientation. A single crystalline, oriented nature was also detected in thicker BT MB (Figure 2a 12). The (001)/(100) preferential orientation of BT NSs and BT MBs was also clearly visible from their X-ray diffraction (XRD) patterns deposited from the alcohol suspension on the Si-single crystalline substrate (Figure S2, Supporting Information). The highest aspect ratio was characterized for BT NTRs, which were nanotextured rods (Figure 2a 7,8), as well as for HAp NRs, which were laterally connected nanothick rods with smooth surfaces (Figure 2a 13,14), both highly crystalline (Figure 2a 9, a 15). Individual HAp NRs are single crystalline structures (Figure 2a 15), with <001> preferential orientation. Within drawn PLLA films, filler particles significantly affected the morphology of the PLLA matrix (Figure 2b). Their surfaces were rough and had patterns that followed the drawing direction (Figure 2b 1–b9). In the case of high aspect ratio BT NTR and HAp NR fillers, surface patterns are formed of highly oriented PLLA fibers, corresponding to crystalline lamella and nonoriented islands, as amorphous parts of the structure (Figure 2b 1–b9). In the case of low-aspect ratio BT NPs, BT NSs, and BT MBs particles, the filler particles were mainly detected inside the film as smaller particle aggregates (evident in the film cross section, Figure 2b 10) or as individual plates inserted inside defects between densely packed oriented lamellae (Figure 2b 11,12). In contrast, individual BT NTRs and HAp NR particles could not be detected as inserted structures, and they might have been more tightly coated with the polymer (Figure 2b 13). The oriented PLLA fibers that they formed were not densely packed, and bulk porosity was identified as their important morphological feature (see film cross-sections in Figure 2b 14,15).

The chain orientation of PLLA influenced by a small quantity of fillers was determined using polarized Raman spectroscopy. The intensity of the peak corresponding to C-COO groups (as relevant structural dipoles) was measured in directions parallel and perpendicular to the film drawing direction, and their ratio was considered an orientation factor. Using this approach, we observed that the presence of fillers particularly enhanced PLLA orientation when small quantities of BT NTRs and HAp NRs were used as fillers (Figure 3h). In these two cases, the enhancement of the PLLA chain orientation relative to nonmodified PLLA chains was found to be statistically relevant, with $p = 0.001$ for PLLA BT NTRs DR5 and $p = 0.05$ for PLLA HAp NRs DR5. The highest orientation factor (2.90 ± 0.05) was obtained for PLLA BT NTRs DR5.

2.2. Filler-Modified PLLA Films—Effect of Fillers on Polymer Crystallization

Filler-modified PLLA, obtained after solvent casting and hot pressing (DR1), resulted in nontransparent films (Figure 3a) composed of an amorphous polymer matrix and dispersed crystalline filler particles (Figure 3a, b). Regardless of the type of filler, which was either tetragonal BT or hexagonal HAp NRs, polymer crystallization took place during the second step when the DRI films were drawn to five times their initial length (DR5, Figure 3b). In most cases, drawing films with 1 wt% BT fillers with different morphologies (Figure 3c) induced crystallization of the polymer into the α′-PLLA phase, which was the same as for nonmodified PLLA (Figure 3d). Despite the small quantity of BT fillers in the PLLA film, the characteristic diffractions of highly crystalline BT are discernible. Moreover, the higher intensities of the (001)/(100) diffractions compared to that of the (110) diffractions, particularly evident for BT MBs and BT NSs, indicate that the 2D BT filler particles were aligned along the PLLA film matrix in the film drawing direction. In
Figure 2. Morphology and structure of filler-modified PLLA films and their components. a) SEM morphology and TEM/SAED structure of filler particles, including BT NPs (a₁, a₂, a₃), BT NSs (a₄, a₅, a₆), BT NTRs (a₇, a₈, a₉), BT MBs (a₁₀, a₁₁, a₁₂), and HAp NRs (a₁₃, a₁₄, a₁₅); b) SEM morphology of surface and cross-section of drawn films modified with 1 wt% of fillers, including PLLA BT NPs DR5 (b₁, b₂), PLLA BT NSs DR5 (b₃, b₄), PLLA BT NTRs DR5 (b₅, b₆), PLLA BT MBs DR5 (b₇, b₈), PLLA HAp NRs DR5 (b₉, b₁₀).
addition to diffraction maxima corresponding to tetragonal BTs, the polymer phase in these films had a maximum at 16.4° corresponding to (200) in α′-PLLA. An exception was the BT NTR filler (with the highest aspect ratio), in which there was an additional diffraction maximum of the polymer at 28.8° (Figure 3d), commonly assigned to partial formation of the β-PLLA phase.\[25,29\] The formation of a mixture of α′- and β-PLLA phases was even more pronounced in the case of PLLA films with HAp NR filler (Figure 3c), for which a high aspect ratio is also characteristic. An additional diffraction maximum at 28.8° (formation of the β-PLLA phase) was observed in the case of low contents of HAp NRs (1 or 5 wt%), while for higher HAp NR contents (10 wt%), only α′-PLLA was detected (Figure 3c). As β-PLLA is less thermally stable than α′,\[23,25\] its presence in BT NTR- and HAp NR-modified PLLA films affected their thermal properties, as detected based on differential scanning calorimetric (DSC) analysis (Figure 3e). The melting peaks of both films that contained a high-aspect-ratio filler exhibited two melting points \(T_{m1}\) at 175°C and \(T_{m2}\) at 178°C, either as two separated maxima or a maximum and a shoulder, and it was

Figure 3. Crystallization of filler-modified PLLA. a) Processing amorphous solvent-casted to crystalline five-times drawn (DR5) films followed by b) drawing-induced polymer crystallization. c) Phase composition of HAp NR-modified PLLA films (1, 5, and 10 wt% HAp NR contents). d) Phase composition of BT-modified films (1 wt% BT NPs, BT NTRs, BT NSs, or BT MBs). e) DSC melting temperature \(T_{m}\) peaks of PLLA in filler-modified films. f) Polarized FTIR spectra with assigned CH\(_3\)-rocking modes of α′- and β-PLLA crystals. g) DSC-determined crystallinity and h) polarized Raman spectroscopically determined molecular orientation of PLLA within filler-modified films; statistical analysis was performed for PLLA without NPs, \(n = 3\), * and ** indicate \(p < 0.05\), \(p < 0.005\), respectively.
shifted for 5–8 °C to lower melting points in comparison with nonmodified PLLA (processed in the same mixture of tetrahydrofuran (THF)/chloroform (CHCl₃) solvents, used as filler dispersant/polymer solvent) and PLLA modified with other fillers (with T_m at 183 °C and higher). Additionally, the contribution of high-aspect ratio fillers to the PLLA phase composition was detected in the polarized FTIR spectra as well (Figure 3f). The CH₃ rocking modes at 923 and 912 cm⁻¹ correspond to α- and β-PLLA crystals, respectively,[24] which were detected in the spectra of PLLA DR5 films modified with BT NTRs and HAp NR fillers, confirming the formation of a mixture of α- and β-PLLA phases. Besides, stretching C=O group vibrations appear as broader, two-maximum bands in case of PLLA films modified with high aspect ratio fillers (NT NTRs and HAp NRs) (Figure S4, Supporting Information). The same was not detected for PLLA films without fillers or PLLA films modified with lower aspect ratio fillers (BT NSs) indicating specific interactions of these groups with unidirectional high aspect ratio filler particles.

The influence of a small filler content on the crystallinity of the polymeric part of the films was determined from DSC data using melting- and cold-crystallization enthalpies (Figure 3g). Initially, we observed that crystallization of a polymer was affected by the THF/CHCl₃ mixture used in solvent casting, and the films were generally more crystalline than those processed using solid-state melting and drawing optimized in our previous studies.[19–21] In addition, the presence of a small quantity of fillers during drawing further modified the crystallinity of PLLA. Statistically relevant differences in PLLA crystallinity in filler-modified films in comparison to nonmodified PLLA were found for films with 1 wt% BT NSs and BT NTRs (p = 0.01). The highest value was obtained for the PLLA BT NS DR5 film (81 ± 9% PLLA phase crystallinity).

2.3. Filler-Modified PLLA Films—Effect of Fillers on Piezoelectricity

The voltage generated in filler-modified PLLA films during their mechanical deformation with ultrasound revealed that the influence of the filler particles was only indirect. Regardless of whether PLLA films were loaded with piezoelectric BT particles characterized by different tetragonality (as evidenced based on Raman spectra (Figure S5, Supporting Information) and tetragonal splitting of diffraction maxima (Figures S2 and S3, Supporting Information)) or nonpiezoelectric HAp NR filler particles, nondrawn DR1 films did not produce any voltage outcome, and voltage was detected exclusively in the case of drawn DR5 films (Figure 4a). It was noted that the piezoelectric properties of PLLA films modified with high-aspect ratio fillers, BT

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Piezoelectric properties of filler-modified PLLA films. a) Comparison of the peak-to-peak (pk-pk) voltage generated of drawn (DR5) and non-drawn (DR1) PLLA films during deformation with ultrasound. b) Piezoelectricity detected for drawn PLLA DR5, PLLA HAp NR DR5, and PLLA BT DR5 films after deformations with 1 MHz (b1) and 80 kHz (b2) ultrasound (inserted graph presents piezo-signal of PLLA DR5 versus background at 1 MHz US). c) Voltage generated by drawn PLLA DR5 films (without or with 1 wt% fillers, including BT NTRs, BT NPs, BT NSs, BT MBs, and HAp NRs) after deformation with 1 MHz (c1) and 80 kHz (c2) ultrasound and their comparison to standard piezo-PVDF film. Statistical analysis was performed relative to PLLA (without NPs), n = 3, *, **, and *** indicate p < 0.05, p < 0.005, and p < 0.001, respectively.
NTRs and HAp NRs, were very similar although, aside from the morphology, these two types of particles have very different structural and electrical properties (Figure 4b). Although the same for the same type of US set-up used for film deformation (1 MHz set-up in Figure 4b1 or 80 kHz set-up in Figure 4b2), the voltage signal shape differs for the same films mechanically deformed by different set-ups (Figure 4b1 vs Figure 4b2). These differences could be assigned to different background signals detected in for these two set-ups (Figure S6a,b, Supporting Information). The amplitude of the US for these two set-ups is similar, as shown for the reference in MHz- (Figure S6c, Supporting Information) and kHz- (Figure S6d, Supporting Information) frequency range. Comparison of the peak-to-peak voltage generated by PLLA films modified with different fillers, including piezoelectric BT NPs, BT NSs, BT NTRs, and BT MBs as well as nonpiezoelectric HAp NRs, revealed that a small quantity of any of these filler particles was able to induce a statistically significant increase in the piezoelectric response of the PLLA matrix (Figure 4c). In general, films produced 2–3 times higher voltage after they were deformed with 1 MHz ultrasound than in the case of their deformation with 80 kHz ultrasound. Relative to a piezoelectric PVDF standard (STDT1-028K, TE connectivity, Germany, uniaxially drawn and poled film with \(d_{33} = 33 \text{ pC N}^{-1}\)) used as a reference, the outcome voltage of the filler-modified PLLA films was 2–5 times lower. Nevertheless, it is important to note that introducing only 1 wt% fillers increased the voltage outcome of nonmodified PLLA up to 20 times.

### 2.4. Piezostimulation with Filler-Modified PLLA Films—Stimulated ROS Formation and Red Blood Cell (RBC) Hemolysis

Since the main idea behind modifying PLLA films with filler particles was to optimize their piezoelectricity for US-mediated cell stimulation, a very important next step was to detect potential side products generated during their interaction, including the formation of ROS, and to investigate their influence on safety. Hence, a previously optimized dihydrorhodamine (DHR) assay was applied.\(^42\) This method is based on the oxidation of an ROS indicator to its fluorescent form and uses vitamin C as an antioxidant and ROS scavenger for detection control (Figure 5a). Since the ROS indicator was added before applying US stimulation, it was able to detect total ROS generated by US as well as by the sonicated samples.

Initially, ROS formation was investigated for free filler particles (Figure 4a), and concentration-dependent ROS production was detected in the case of BT particles (Figure 5a2–a3). Their initial capacity to produce ROS (without US) was further increased during US stimulation (Figure 5a2–a3).

A statistically relevant increase in ROS production relative to ROS produced by US without particles was mainly confirmed for the highest (500 \(\mu \text{g mL}^{-1}\)) BT particle content. Another interesting observation was that ROS production was also related to BT particle morphology. The ability of BT particles to produce ROS increased in the following order: BT MBs < BT NPs < BT NSs < BT NTRs (Figure 5a1–a3). The highest amount of ROS was detected for rod-like BT NTRs with a high aspect ratio and nanostructured surface, and it was comparable to UV light-treated 5% \(\text{H}_2\text{O}_2\) used as a positive control (Figure 5a1). In contrast to BTs, ROS production with HAp NR particles was not significant and was comparable to the ROS produced solely by sonicated medium without particles (Figure 5a2).

When ROS production was investigated in DR5 filler-modified PLLA films, the situation was very different (Figure 5b). Since each film contained only 1 wt% of a filler, any produced ROS could be mostly assigned to the PLLA matrix. We observed that all of the tested PLLA films were able to produce very low amounts of ROS that were comparable to nonmodified PLLA (Figure 5b). An increase was observed when the films were sonicated with US. However, the observed changes were comparable to the ROS detected in the medium sonicated without films. The observed differences between the ROS detected for PLLA films and for the sonicated medium without any films were not found to be statistically relevant.

Further comparative analysis of ROS formation during stimulation of filler-modified films with 1 MHz and 80 kHz US was performed in the presence of RBCs (Figure 5b). The experimental setup used for stimulation was the same as for the stimulation of adherent cells (Figure 6a1,a2). The main difference was that the nonadherent RBCs floated in the media around the films during the stimulation. In the positive control, stimulation with 80 kHz US evidently generated more ROS than 1 MHz US, for which ROS was comparable to the nonstimulated control. However, ROS detected for the filler-modified films remained low even after both types of US stimulation, and there were no statistically relevant differences between the two frequencies. Along with ROS production, we also measured RBC hemolysis during US stimulation (Figure 5b4). These measurements revealed up to a few percent RBC hemolysis during US stimulation with filler-modified films with no significant difference from the film-free RBC control. Moreover, there was no difference in hemolysis between the sonicated RBCs and nonmodified RBCs, which confirmed that stimulation with the two selected US sources was not aggressive to the cells and could not damage the membranes of RBCs.

### 2.5. Piezostimulation with Filler-Modified PLLA Films—Stimulated Growth of Keratinocyte (HaCaT) Cells

Skin HaCaT cells were selected as the adherent cell model. They were seeded directly on the filler-modified PLLA films (both DR1 and DR5) and adhered to their surfaces. In this way, the cells were in intimate contact with the piezoelectric surface and directly influenced by the periodic changes in the polarization obtained during the stimulation.

Films with adherent cells were placed into 24-well plates and subjected to stimulation in two experimental setups: in the first one, we applied 80 kHz US in continuous mode inside a US bath (Figure 6a1), and in the second one, we applied 1 MHz US in pulsed mode using a US transducer (in contact with a Petri dish filled with water, similar to inside the bath) (Figure 6a2). The continuous 80 kHz stimulation was longer (20 min), and the pulsed 1 MHz stimulation was shorter (3 min). Both were performed daily and repeated in three cycles following cell incubation. Immediately before and after each stimulation cycle, we recorded the plates with an IR thermal camera to...
obtain information about the temperature changes induced by US stimulation and to detect the distribution of the heat (as a rough approximation of distributed US) along the plates. The recorded temperature changes during the stimulation inside the US bath at 80 kHz were higher, and the plates were heated to \( \approx 10^\circ\text{C} \) (Figure 6a1). In the case of pulsed and shorter 1 MHz stimulation, the stimulation increased the temperature by \( \approx 3^\circ\text{C} \) (Figure 6a2). In both cases, the heat was mostly distributed evenly along the plates, meaning that the films placed at different positions in the plates were equally stimulated. Moreover, the temperature in the walls with films and cells remained below the cell incubation temperature throughout all US stimulations, so any harmful effects of heating on the cell could be excluded.

Figure 5. Reactive oxygen species (ROS) during piezostimulation. a) The principle of measurement with vitamin C ROS scavenger as detection control (a1). ROS detection for different concentrations (500, 50, and 5 \( \mu\text{g mL}^{-1} \)) of NPs (BT NPs, BT NTRs, BT NSs, BT MBs, and HAp NRs) with and without US piezostimulation (80 kHz) (a2). b) ROS detection in different filler-modified PLLA films (with 1 wt% of fillers) with and without US piezostimulation (80 kHz) (b1), ROS detection during stimulation of RBC cells with PLLA 1 wt% composites (1 MHz or 80 kHz ultrasound) (b2) as well as RBC hemolysis detection after piezostimulation (b3). ROS positive (ROS\(^+\)ctr) and negative (ROS\(^-\)ctr) were ROS detected in HEPES and 5% H\(_2\)O\(_2\), while hemolysis positive (Hem\(^+\)ctr) and negative (Hem\(^-\)ctr) controls were RBC in H\(_2\)O and RBC in HEPES; statistical analysis was done for ROS formation and hemolysis by filler particles or films relative to ROS detected in HEPES buffer without NPs or films, \( n = 3 \), ** and **** indicate \( p < 0.005 \) and \( p < 0.0001 \), respectively.
On the surface of PLLA films, HaCaT cell initial adhesion as well as further growth were affected in a way that most of the filler-modified films promoted both of these steps, and generally, cells were growing faster than on the surface of nonmodified PLLA (Figure 6b1,b2). The only exception was the BT NP-modified PLLA film, for which promotion of cellular growth was not detected. It was very similar to their growth on the surface of nonmodified PLLA DR5. On the other hand, direct comparison between US-stimulated and nonstimulated films (Figure 6b1,b2) showed that US promoted cellular growth only at the surface of BT NTR- and HAp NR-modified DR5 PLLA films, for which statistically relevant differences in cell growth were detected. This means that only the filler-modified films with a high aspect ratio were able to provide US-mediated stimulation of cellular growth. It was also interesting to note that 80 kHz US and 1 MHz US affected cellular growth very similarly, yet the differences in 1 MHz-stimulated cellular growth were more pronounced ($p < 0.0001$ for 1 MHz and $p = 0.001$ for 80 kHz for comparing cellular growth with and without US (US+ vs US−)). Importantly, growth was very similarly promoted on the DR5 and DR1 films (Figure S7, Supporting Information), which means that the contribution of the piezoelectricity of the films to cell proliferation was not the dominant effect. Thus, the observed differences in cell proliferation were a consequence of a combination of US stimulation and filler-induced film bioactivity.

Figure 6. Piezostimulation of HaCaT cells. a) The spread of the heat and temperature change before and after stimulation of cells on top of PLLA composite films using 80 kHz (a1) and 1 MHz (a2) ultrasound (US) for three daily stimulation cycles; proliferation of cells on the surface of PLLA composites (with 1 wt% BT NPs, BT NTRs, BT NSs, BT MBs, or HAp NRs) with and without stimulation assisted with 80 kHz (b1) or 1 MHz (b2) US. Statistical analysis was performed for cells grown on PLLA composite films relative to PLLA films (without NPs) as well as for comparisons of cellular growth with (US+) and without US stimulation, $n = 3$, **, ***, and **** indicate $p < 0.005$, $p < 0.001$, and $p < 0.0001$, respectively.
2.6. Piezostimulation with Filler-Modified PLLA Films—Stimulated Cytoskeleton Response

The major contribution of the piezoelectricity of filler-modified films was in promotion of the cytoskeleton response (Figure 7).

Direct comparison confirmed that piezoelectric (DR5) films significantly promoted the formation of actin filaments in the cells after exposure to US, while nonpiezoelectric (DR1) films did not (Figure 7a). A particularly strong contribution to actin filament formation was detected in PLLA DR5 modified with

![Graph and images showing actin filament formation](image)

**Figure 7.** HaCaT cell cytoskeleton response to US-mediated piezostimulation. a) Actin filament formation in cells attached to the surface of filler-modified PLLA films (with 1 wt% of different fillers, including BT NPs, BT NTRs, BT NSs, BT NCc, and HAp NRs). b) Influence of the piezoelectricity and piezostimulation on cellular shape- the cells grown to the surface of nonpiezoelectric PLLA DR1 and piezoelectric PLLA DR5 as well as grown onto filler-modified PLLA films. c) Unidirectional elongation and alignment of cells to drawing directions in the case of PLLA NTRs and PLLA HAp NR films. Statistical analysis was performed for actin filament formation per cell on the surface of piezoelectric (DR5) films relative to their nonpiezoelectric (DR1) counterparts, \( n = 3 \), * and **** indicate \( p < 0.05 \) and \( p < 0.0001 \), respectively.
BT NPs, BT NTRs, and HAp NRs, while films modified with BT NSs and BT MBs retained actin filament formation similar to that of nonmodified PLLA. Stimulation with 1 MHz US particularly promoted actin filaments in cells on the surface of piezoelectric filler-modified PLLA films with a high aspect ratio (PLLA BT NTRs DR5 and PLLA HAp NRs DR5) (Figure 7a) and enabled significant enhancements in their formation in comparison with 80 kHz US stimulation (Figure 7a).

The contribution of US-mediated piezostimulation to the development of the HaCaT cell cytoskeleton was also observed in the cellular shape and distribution of actin filaments inside the cytoskeleton. The cells grown on the surface of nonpiezoelectric PLLA DR1 and piezoelectric nonmodified PLLA DR5 films mostly exhibited the usual cuboidal shape, typical for HaCaT cells (Figure 7b1,7b2). After stimulating them with US, the cells on the surface of PLLA DR1 films started to form filopodia (Figure 7b1), while those at the surface of PLLA DR5, in addition to forming filopodia, changed their shape (Figure 7b2). The unidirectional elongation of cells was even more pronounced in filler-modified PLLA films, and except for the cells on PLLA BT NS DR5 films, which were quite similar to cells on nonmodified PLLA DR5, cells on other filler-modified PLLA DR5 films had a very elongated shape (Figure 7b3–7b8). Interestingly, the largest morphological change was obtained for cells on the surface of high aspect ratio filler-modified films (PLLA BT NTRs DR5 and PLLA HAp NRs DR5) stimulated using 1 MHz US. The elongation direction of these cells was also highly aligned with the drawing directions of the films on which surface they were growing (Figure 7c1–c3). Such alignment was not detected for the cells on the surface of nonmodified PLLA films stimulated with 1 MHz US (Figure 7c3). Together with cell alignment, actin filaments were also aligned with the cell elongation direction as well as with the direction of film drawing. The alignments followed the drawing direction of films as well as the direction of PLLA dipole orientation.

3. Discussion

Mimicking biophysical processes that naturally occur during the self-repair of wounds and damaged tissues inside a living organism, including the role of endogenous electrical fields in electrostimulated regeneration, provides many possibilities for designing the next generation of medical devices with regeneration-stimulating capacity. The paradigm of US-activated piezostimulation, when US is used to mechanically deform a piezoelectric structure and provoke a direct piezoelectric effect producing electrical polarization, which in turn stimulates different phases in cellular life (adhesion, proliferation, differentiation, migration, etc.), is a relatively new branch of biomedical research. As a new strategy that offers an option for remotely stimulated recovery, this process carries great potential for significant advancement in regenerative medicine. Particular benefits are expected from novel generations of piezoelectric biomaterials that are biodegradable, biocompatible, and implantable, which make them highly suitable for applications inside living organisms, including PLLA.

Tuning the piezoelectricity of PLLA is considered to be strongly dependent on the possibility of controlling the crystallinity and molecular orientation. In addition, the structural and morphological properties of PLLA are equally important. From that standpoint, it is very important to control the very beginning phase of its processing, as it will affect polymer crystallization in later phases. Therefore, different fillers, characterized by high crystallinity and different aspect ratios, were investigated for their ability to act as nucleating agents (Figure 1). They were introduced inside the polymer matrix at the very beginning, when amorphous DR1 films were formed (Figure 3). To achieve good homogeneity, it was critically important to disperse the fillers inside a viscous polymer solution, which prevented their aggregation during solvent casting. Another important point was that in this phase, the fillers did not induce any polymer crystallization (Figure 3), which would result in randomly oriented crystallites. Their role in the crystallization process started in the second phase when the DR1 film was drawn (DR5). Drawing-induced crystallization of the melted DR1 film initiates oriented semicrystalline structures (Figure 3). With the help of fillers, the percentage of crystallization of these structures and their molecular orientation were significantly improved (Figure 3). For nonmodified PLLA DR5, all filler-modified PLLA DR5 crystalized into a metastable $\alpha'$-PLLA phase, which has been previously shown to be commonly formed at $T < 100^\circ C$. Previously, it was observed that the presence of BT and HAp NRs may contribute to partial $\beta$-PLLA phase formation. Here, we show that the formation of $\beta$-domains during drawing-induced crystallization is particularly promoted by high-aspect ratio fillers (Figure 1). As $\alpha$ and $\beta$-domains can coexist within multidomain crystallites when $\beta$-domains are usually smaller, with higher disorder, lack of symmetry and more oriented, high-aspect-ratio filler particles promoted their cocrystallization together with $\alpha'$. As we observed, regardless of the difference in chemistry, crystal structure, and electrical properties, both HAp NRs and BT high aspect ratio fillers had dominantly high contributions in enhancing oriented PLLA crystallization in comparison to all other filler particles (Figure 3). In our opinion, the critical events for oriented PLLA crystallization take place at the interface between filler particles and polymer matrix and this process strongly depends on interface interactions. In general, we have observed that addition of any highly crystalline fillers, HAp, or BT, inside PLLA matrix promotes polymer crystallization indicating their role as nucleating agents in this process. In most cases, lower aspect ratio fillers (nanoparticles, sheets and blocks), did not affect the type of PLLA crystalline phase and the polymer crystallizes into $\alpha'$-phase which is thermodynamically the most stable and commonly formed at used temperature (also without application of fillers). The main role of the solid surface of filler particles here was to act as nucleating agent which promotes crystallization process, reinforce larger fraction of amorphous domains to arrange into ordered crystaline structure, consequently contributing to increased polymer crystallinity. However, in case of filler particle with unidirectional high aspect ratio (nanotextured or flat rods), situation was very different. In addition to promotion of $\alpha'$-phase crystallization, their oriented surface also initiated $\beta$-phase PLLA formation. The surface of unidirectional high aspect ratio filler particles was able to interact with PLLA molecular chains consequently initiating oriented crystallization of highly ordered
The PLLA phase. We were not able to detect hydrogen bonding between PLLA and HAp which was previously suspected to be the reason for HAp-induced PLLA phase formation. Therefore, we assume that electrostatic interactions between cations (Ga$^{3+}$ or Ba$^{2+}$) along uniaxially oriented HAp or BT surface with C=O groups in PLLA chains initiate forming more ordered β structure. The assumption is mainly based on the changes of C=O group stretching vibrations observed only in case of PLLA films modified with uniaxially oriented high aspect ratio HAp or BT filler particles (Figure S4, Supporting Information). Very similar interactions exist in hierarchically organized bone structure, where ordered collagen fibrils are aligned with uniaxially oriented high aspect ratio mineral particles. In bone, mineral particles are present as anisotropic structures with the longest axis aligned to the length of collagen fibers which are bend around mineral particles (known as interfacial interlocking). These interactions are essential for the load transfer and they have important role in keeping the structural stability and preventing piezoelectricity of bone.

In contrast to the common cases when piezoelectricity comes from the fillers, the piezoelectricity of filler-modified PLLA DR5 films presented here originated from the polymeric matrix. Together with crystallinity and orientation, the fillers had an additional role related to the morphology of PLLA films (Figure 2). Since oriented fiber-like structures obtained using the high aspect ratio particles enabled higher porosity, their piezo-response was not dominantly high in comparison to other fillers. It was very important that the improvement of PLLA piezoelectricity (and associated properties) was induced using a low quantity of fillers (just 1 wt%). In contrast to HAp NRs, BT particles produced ROS during interaction with US, and the production was concentration dependent (Figure 5). Within the filler-modified PLLA film, ROS production during the interaction of small fillers with US was also very low and did not increase with film piezoelectricity (Figures 4 and 5). US-mediated ROS production by small BT nanoparticles has been detected previously and has been connected to the possibility of inducing acute hyperproduction of ROS, which may enable apoptosis of cancer cells. However, high ROS levels, particularly during external stimulations, induce oxidative stress, which may cause permanent damage to the keratinocyte cellular system, and chronic exposure to ROS may lead to different pathological conditions. Local heating of polymers during stimulation with US is not fully understood, and some studies suggest that it may differ depending on their crystallinity and structure.

From that standpoint, since ROS production in US-mediated piezostimulation with filler-modified PLLA films was low and detected local heating did not induce high temperatures, the stimulation has been confirmed to be a mild process without destructive effects in cells (Figure 5), a property very important for ultrasound-activated biomaterials.

An evident increase in PLLA piezoelectricity induced by a small quantity of fillers (Figure 4) was able to shift the voltage outcome of PLLA films after mechanical deformation with US to the range that corresponds to the endogenous potential (150–1200 mV) required for wound healing. The selection of US frequency used for film deformation had important role to their response. In general, US waves have two physical effects in biological systems—thermal and mechanical. Thermal effects mainly depend on transmitted power, while mechanical mainly depend on negative pressure ($P_{\text{US}}$) and frequency ($f$) (mechanical index MI = $P_{\text{US}}/f^2$). The application in regeneration thermal/physical effects are optimized to provide middle heating with nondestructive mechanical stimulation. This gets much complicated when piezoelectric biomaterial is involved, because both type of effects will be additionally affected by the interactions between biomaterial and US. In addition to thermal and mechanical effects, piezoelectric material mechanically deformed with US will bring electrical effects as third stimulating parameter. In case of 80 kHz, MI is intermediate, electrical output is lower but thermal index is also low. It was also interesting to notice that stimulation using this frequency enables antibacterial effects without negative effects on human cells. Selection of a frequency from MHz region is mainly related to its ability to provide higher penetration depth inside human tissue and have higher potential for the use in stimulation-related regeneration inside organism. As we observed, for MHz frequency both thermal and mechanical effects are minimal, while electrical effects match the natural endogenous electrical effect, which is a promising combination for effective regeneration. Endogenous piezostimulation was shown to have critically important contribution to regeneration, as it was observed for the case of stimulated peripheral nerve regeneration. While the effect of electrostimulation on human keratinocytes is well studied with clear evidence for promoted proliferation, differentiation, and migration, the contribution of piezostimulation is widely unexplored. The detected response of HaCaT cells to US-mediated piezostimulation was a combination of a contribution of surface properties (chemistry, topography, and porosity), mechanostimulation (cells-US interactions), and piezostimulation (electrical polarity-induced cell stimulation) (illustrated in Figure 8). The surface properties of PLLA films primarily affected cell attachment (Figure 8a). In general, adding fillers to PLLA films increased their bioactivity, and cells favored attachment to their surface more than to nonmodified PLLA. Initial HaCat cell adhesion was particularly promoted on the surface of PLLA films with higher porosity, formed by high-aspect-ratio filler particles. Porous structures offer a larger contact area, consequently promoting the first phase of cell-material interaction related to cell adhesion, as was observed for osteoblasts grown on aligned and porous lamellar apatite/ BT structures. Mechanostimulation during interactions of cells with US had the main role in affecting cell proliferation (Figures 6 and 8b). US stimulation significantly promoted cell proliferation, which was the case for both piezoelectric DR5 and piezoelectric DR1 films, and it was a consequence of interactions with US rather than a contribution of piezostimulation (Figure 6). Low-intensity US transmits acoustic waves through cells and tissues, providing noninvasive physical stimulation with repair/regenerative potential. Particularly during interactions with keratinocytes, US stimulation promotes proliferation and cell migration, consequently contributing to regeneration and wound healing. The contribution related to piezostimulation has been observed to affect cytoskeleton organization (Figures 7 and 8c). Previously, it was observed that intracellular stimulation of skeletal muscle and...
dermal fibroblast cells (internalized with piezoelectric boron nitride nanotubes stimulated with US) induces actin overexpression.[62,63] US-mediated piezostimulation promoted cell differentiation, which formed aligned myotubes on patterned surfaces.[52] Extracellular stimulation of cells directly attached onto the PVDF piezoelectric surface has been observed to result in differentiated multipolar neural cells with extensive axon and dendrite formation, without alignment in the preferential direction.[64] Interestingly, in the case of piezoelectric PLLA films, US-mediated stimulation significantly increased actin filament formation specifically at the surface of highly oriented piezoelectric DR5 films. Cells on piezoelectric films were intensively polarized and aligned in a specific direction that corresponded to the film drawing direction and direction of the PLLA dipole orientation. Among others, actin has a role in cell migration and regulation of cell shape, particularly in response to external, mechanical, or electrical stimuli. Mechanostimulation during cell interactions with US waves activated their cytoskeletal response, as evidenced by randomly oriented filopodia formation. The piezostimulation, induced by the US-mediated electrically polarized film surface, further affected actin filaments, which was a consequence of piezoelectrically promoted electrotaxis, as cells tend to migrate and elongate in specific polarization directions.

In wound healing and tissue regeneration, directional cell migration has a particularly important role. In a cascade of different highly controlled events, the actin cytoskeleton regulates extracellular matrix formation and enables mechanical support for mobilization of various cells involved in this process. In this context, US-mediated piezostimulating biomaterials, such as oriented piezoelectric PLLA, might be applied as highly functional materials that would guide the cells over a wound or a crack to seal it more efficiently. Particular advances might be expected in designing active, implantable medical textiles able to stimulate postsurgical recovery. Application of uniaxially oriented high aspect ratio filler particles as fillers for designing structural and piezoelectric properties of PLLA as very perspective organic piezoelectric biomaterial for the future.

4. Conclusion

Modification of PLLA with a low content (1 wt%) of high-aspect-ratio, piezoelectric or piezoelectric filler particles was found to be a very effective strategy for tailoring its piezoelectric property. The approach optimizes the design of PLLA as a US-activated biomaterial and shifts its voltage output to the range of endogenous potential required for wound healing. Consequently, US-mediated piezostimulation promotes skin keratinocyte cell behaviors, including orientation and directional migration, which are very important for regeneration. The material is envisaged as a very functional tool particularly applicable after implantation for promoting wound regeneration inside living organisms.

5. Experimental Section

**Materials:** Calcium nitrate pentahydrate (Ca(NO3)2.5H2O) (Sigma- Aldrich, Germany), ammonium dihydrogen phosphate (NH4H2PO4) (Sigma-Aldrich, Germany), urea (CO(NH2)2) (Alfa Aesar, Germany), Bi2O3 nanopowder (Alfa Aesar, 99.9%), TiO2 (Degussa P25, 99.5%), NaCl (Merck, 99.5%), KCl (Supelco, 99.5%), BaCO3 (Alfa Aesar, 99.99%), ultrapure water (with 18.2 MΩ cm resistivity, obtained by Purelab Option Q7, ELGA), poly-L-lactic acid polymer L207 S (Evonik, Germany), chloroform (Sigma-Aldrich, Germany), THF (Sigma-Aldrich, Germany), dihydrodorhamidine 123 (DHR, >95%, Sigma-Aldrich), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Fisher Scientific, Taiwan), sodium chloride (Carlo Erba Reagents, Germany), sodium hydroxide (NaOH, Sigma-Aldrich), Dulbecco’s modified Eagle’s medium-high glucose (DMEM, Sigma-Aldrich), foetal bovine serum (FBS, Gibco, US), penicillin–streptomycin antibiotics (1:1, Gibco, USA), Dulbecco’s phosphate buffered saline (DPBS, Sigma-Aldrich, USA), TrypLE select (Gibco, USA), poly-L-lysine solution (mol wt. 70,000–150,000, 0.01%, sterile-filtered, 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) were used in this study. All of the chemicals and reagents were of analytical grade.

**HAp NR Synthesis:** HAp NRs were synthesized using a urea-assisted homogenous precipitation method.[41,64] Aqueous solutions of Ca(NO3)2, NaOH (9.6 mg mL⁻¹, 50 mL) and NH4H2PO4 (2.3 mg mL⁻¹, 50 mL) were mixed and heated to 80 °C, which was followed by the addition of a 12 wt% solution of urea (10 mL), and mixing at the same temperature was continued during the next 3 h. The precipitate was
aged in supernatant at room temperature for the next 15 h, followed by centrifugation and washing in water. The precipitate was kept wet inside a centrifuge tube at 4 °C for further use in processing PLLA films.

**BT Synthesis:** BT filler particles were synthesized using a template-assisted approach.[40–48] In the case of BT NPs and BT NTRs, the template was a hydrothermally synthesized TiO$_2$ NWS precursor,[46] which was mixed with Ba(OH)$_2$ 8H$_2$O, and the mixture was further hydrothermally reacted to form the desired BT morphology. For the synthesis of BT NPs, 0.51 g of as-prepared TiO$_2$ NWSs were dispersed in 70 mL of 0.2 M Ba(OH)$_2$ 8H$_2$O aqueous solution and sonicated for 5 min. The dispersed dispersion was transferred into a 100 mL Teflon-lined autoclave and heated at 240 °C for 12 h. Similarly, BT NTRs were synthesized from 0.15 g of TiO$_2$ NWSs and 0.05 M Ba(OH)$_2$ 8H$_2$O solution at 210 °C for 85 min. After the reaction, the precipitates were collected and washed with 0.2 M HCl solution and deionized water and dried at 70 °C overnight. In the case of BT NSs, the templates were Bi$_4$Ti$_3$O$_{12}$ NSs.[40] In a hydrothermal process, 0.12 g of sodium oleate was dissolved in 160 mL of 12 M NaOH aqueous solution, and then 2.198 g of BiCl$_3$ 2H$_2$O and 0.4 g of Bi$_4$Ti$_3$O$_{12}$ NSs were added and stirred for 1.5 h. The final dispersion was poured into a 200 mL Teflon autoclave and heated at 240 °C for 20 h. Finally, the precipitate was washed repeatedly with 2 M HNO$_3$ solution and ethanol, after which it was dried at 70 °C overnight. BT MBs were synthesized through topochemical conversion of Bi$_4$Ti$_3$O$_{12}$ template platelets in molten (NaCl/KCl) salt at 900 °C.[40,76,68] BaCO$_3$ and salts (NaCl/KCl) were first thoroughly ground to fine particles, and then the Bi$_4$Ti$_3$O$_{12}$ microblocks were gently admixed. The ratio between the salts and the reagents was as follows: KCl: NaCl: Bi$_4$Ti$_3$O$_{12}$: BaCO$_3$ = 39: 39: 1: 10. The powder blend was heated to 900 °C, annealed at this temperature for 2 h and slowly cooled (1 °C min$^{-1}$) to room temperature. After removal of the salts by dissolving in water and air-dried by sonication in 2 M HNO$_3$ (5 min) along with elimination of the acid remains by water washing, followed by drying (in air). The details on the synthesis of different templates are provided in Method S1 (Supporting Information).

**Solvent-Casted Filler-Modified PLLA Films:** PLLA was dissolved in CHCl$_3$ (20 mL, 100 mg mL$^{-1}$) by mixing with a magnetic stirrer (500 rpm) for 2 h at room temperature. Fillers were dispersed in THF (20 mg in 5 mL) using an ultrasonic bath (37 kHz, 100% amplitude, 5 min). The dispersion was added to completely dissolved PLLA solution, and mixing was continued for the next 60 min. The mixture was transferred to a glass Petri dish (diameter 10 cm) and left to dry in a fume hood overnight.

**Drawn Filler-Modified PLLA Films:** The solvent-casted films were further processed into nonpiezoelectric (DRI) and piezoelectric (DRS) films using a previously optimized protocol.[9–21] DRI films were formed when small parts of solvent-cast films (0.7 g) were melted inside an aluminum mold (3×3 cm and 100 µm thick) at 250 °C (incubator Kambic, Slovenia) for 15 min, pressed under 500 kN (manual lab press, Weber, Germany) for 3 min and immediately quenched in a cold ethanol/water mixture (30:70:30, %). DRS films were formed when DRI films were cut into dumbbell-shape films and uniaxially stretched with a homemade tensile stretcher to a draw ratio of 5 at a temperature above the glass transition (90 °C) using a drawing rate of 40 mm min$^{-1}$. As reference, PLLA films without fillers were prepared using the same protocol.

**Physico-Chemical Characterization:** XRD analysis of powders (HAp NRS and BTs) was performed in BRUKER AXS D4 ENDEAVOR (Cu Kα, 1.54 Å, 2θ range 10°–60°, 0.04° step size with 5 s time of capture) and filler-modified PLLA films in EMPIREAN (2θ range 10°–60°, 0.026° step size with 500 s time of capture) to identify crystalline phases. Crystallinity was determined using DSC (NETZSCH STA 449 (jupiter) thermal analyzer) in an Ar/O$_2$ atmosphere (40/10). The samples were heated in the range 40–200 °C using a 20 °C min$^{-1}$ heating rate. The crystallinity was determined using the following equation: Xc (%) = $\frac{\Delta H_m - \Delta H_c}{\Delta H_{100}}$, where $\Delta H_m$ and $\Delta H_c$ correspond to enthalpies of cold crystallization and melting, respectively, while $\Delta H_{100}$ is 93.6 J g$^{-1}$ and presents a theoretical value for 100% crystalline PLLA films. Polarized Raman spectroscopy measurements were performed using NTEGRA Spectra NT-MDT with a polarized 488 nm laser source in the range from 100 to 3200 cm$^{-1}$ with 100 s of acquisition and 5 mW laser power. The spectra were recorded in the horizontal and vertical orientations of the polarizer relative to the film drawing direction. The normalization was performed relative to the CH$_3$ asymmetric bending mode at 1454 cm$^{-1}$. The orientation factor (R) was calculated as a ratio between the intensities of the C-OO group peak (at 875 cm$^{-1}$) recorded in two different directions. The surface morphology of fillers and filler-modified PLLA films was investigated using scanning electron microscopy (JSM-7600 F, Jeol Ltd., Tokyo, Japan). The samples were sputtered with a several nm thick gold layer and observed using an SEM detector at 5 kV and a 6 mm working distance. The structural characteristics of filler particles were investigated using a transmission electron microscope (JEOL JEM-2100, Jeol Ltd., Tokyo, Japan) operated at 200 kV with a beryllium double-tilt specimen holder to determine the crystal structure.

**US-Mediated Piezostimulation:** Two experimental setups were used for deforming films with ultrasound. In the first case, the films were stimulated with pulsed 1 MHz ultrasound with on/off intervals of 1:10 s and 0.8 W cm$^{-2}$ power. The US transducer was placed on the bottom of a glass Petri dish with a 20 cm diameter filled with 100 mL of ddH$_2$O. The contact between the transducer and a glass was supported by a thin layer of US transducing gel (Ultragel Hungary 2000 Kft., Hungary). In the second set up, the films were stimulated in an ultrasonic bath using continuous 80 kHz ultrasound and 30% intensity.

**Piezoelectric Measurements During US Stimulation:** The voltage output was measured directly when films were subjected to mechanical stimulus using 80 kHz and 1 MHz ultrasound. Before measurements, both sides of the films were sputtered with gold electrodes, connected to cables and impregnated against water intrusion. Cables were routed through a piezo-film to a Keysight MSOX3034T oscilloscope to record the signal. The measurements were performed in at least three different films.

**ROS Detection for US-Stimulated Films and Filler Particles:** A DHR assay was applied to detect ROS generation during US stimulation.[42] Testing was performed for dispersed filler particles (0.5, 0.05, and 0.005 mg mL$^{-1}$) as well as for filler-modified PLLA films (≤5 × 5 mm) both in HEPES buffer (modified with NaCl (0.9%) and NaOH (1 mM) to pH = 8) inside 96-well plates. In both cases, there was a control sample group with added vitamin C as an ROS scavenger (40 µL well$^{-1}$, 1 mg mL$^{-1}$). The same groups, with and without vitamin C, were prepared for stimulation with 80 kHz and 1 MHz US as well as a control group without US. In all groups, the ROS level was measured with 80 kHz and 1 MHz US stimulation and determined by a confocal laser scanning microscope. Additional controls were HEPES medium without filler particles as a negative control and H$_2$O$_2$ (5%) preirradiated with UV light as a ROS positive control. After stimulation, the samples were incubated for 1 h at 37 °C, which was followed by measuring fluorescence at 490/530 nm $E_L/E_B$. All samples were tested in triplicate.

**RBC Hemolysis During US Stimulation:** RBCs were isolated from whole sheep blood (BioSap SO defibrinated and reoxygenated sheep blood, 50 mL) and diluted in HEPES buffer (modified with NaCl (0.9%) and NaOH (1 mM) to pH = 8) to a final concentration of 2.5% mL$^{-1}$. Filler-modified PLLA films (≤5 × 5 mm) were covered with 100 µL of RBCs in HEPES inside three 96-well plates for stimulation at 80 kHz, stimulation at 1 MHz and nonstimulated reference. After stimulation, the plates were incubated at 37 °C for 2 h and centrifuged (500 rcf, 5 min), and supernatants were evolved for hemoglobin release by measuring the absorbance at 540 nm (Synergy H1, Biotec). The results were normalized to RBCs in HEPES and RBCs in ddH$_2$O as hemolysis-negative and hemolysis-positive controls, respectively. All samples were tested in triplicate.

**Piezostimulated HaCat Cell Growth:** HaCat human keratinocyte cells (ATCC PCS-200-011) were confluent grown in 6-well plates in full DMEM growth medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin). Before testing, nonmodified and filler-modified PLLA films (1 × 1 cm) were immersed in poly-L-lysine solution (300 µL), which was followed by washing with full DMEM. Washing with full DMEM was also used as a film sterilization method. The films were seeded with HaCat cells (diluted to 1:8 in full DMEM) inside 24-well plates and incubated at 37 °C and 5% CO$_2$ (MCO-19AIC(UV)-PE, Panasonic). After 24 h of incubation, the films were replaced in new 24-well plates, and
cell attachment was measured using Presto Blue Cell Viability Reagent (following the provided protocol by using 10 wt% reagent per well, incubation of samples with reagent for 1 hour in an incubator and measuring fluorescence at 560/590 nm $E_{Fm}$). After detecting viable cells on the films, they were washed with Presto Blue (using DPBS), added to fresh full DMEM and subjected to piezostimulation with 80 kHz US or 1 MHz US or left without stimulation. In all three cases, cells were transferred to a CO2 incubator and incubated for an additional 24 h. The protocol including growth measurement and stimulation was repeated for 3 days. Each stimulation step was recorded with an IR thermocamera (FLIR E8) immediately before and after stimulation to detect the stimulation-induced increase in the temperature inside a 24-well plate. Analysis was performed in at least two independent experiments, each time in three parallel experiments.

**Actin Filament Measurements:** Cells grown on filler-modified and nonmodified PLLA films, subjected to 3-day stimulation with 80 kHz or 1 MHz US or remaining nonstimulated, were further analyzed for actin filament formation. The staining protocol provided by the producer included fixing cells with paraformaldehyde (3.3% solution in DPBS), permeabilization with Triton X 100 (0.5% solution in DPBS), washing with DPBS, and staining with TRITC (1 µL mL$^{-1}$) for actin filament detection. Contra staining was performed using DAPI (5 µL mL$^{-1}$ stock in Hank’s balanced salt solution). RP-stained actin filaments were detected at 584/562 nm $E_{Fm}$ while DAPI-stained DNA was measured at 355/346 nm $E_{Fm}$ (Synergy H1, Biotec). Totally formed actin filaments were normalized to total cell number and presented as actin filaments per cell. Stained films with stained cells were placed on a cover glass and observed under a fluorescence inverted microscope (Eclipse Ti-U inverted microscope, Nikon). Analysis was performed in at least two independent experiments, each time in three parallel experiments.

**Statistical Analysis:** The results are presented as the mean values of at least three measurements ($n = 3$) and are indicated with standard deviation (SD). Differences between groups were assessed by one-way ANOVA (GraphPad 9.0 Software) with a confidence level of 95% and $p < 0.05$. The analyses were performed in at least two independent experiments.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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