Supplementary Material

Piezoelectric nanocomposite bioink and ultrasound stimulation modulate early skeletal myogenesis

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Gene	Primer sequence (5' – 3')
GAPDH	Fw: CGACTTCAACAGCAACTCCCACTCTTC Rev: TGGGTGGTCCAGGGTTTCTTACTCCTT
MYOD1	Fw: TCTCCACAGACAGCCCCGC Rev: GGTTCCCTGTTCTGTGTCGCTT
MYOG	Fw: CCCATGGTGCCCAGTGAA Rev: GCAGATTGTGGGCGTCTGTA
CCND3	Fw: CGAGCCTCCTACTTCCAGTG Rev: GGACAGGTAGCGATCCAGGT
CDKN1A	Fw: CGAGAACGGTGGAACTTTGAC Rev: CAGGGCTCAGGTAGACCTTG
MYF5	Fw: TGAGGGAACAGGTGGAGAAC Rev: AGCTGGACACGGAGCTTTTA
ACTA1	Fw: ATGGTAGGTATGGGTCAG Rev: GATCTTCTCCATGTCGTC
CSRP3	Fw: TGGGTTTGGAGGGCTTAC Rev: CACTGCTGTTGACTGATAGG
MYH2	Fw: GCAGAGACCGAGAAGGAG Rev: CTTTCAAGAGGGACACCATC
MYH4	Fw: GAAGGAGGGCATTGATTGG Rev: TGAAGGAGGTGTCTGTCG
ACTN2	Fw: TCATCCTCCGCTTCGCCATTC Rev: CTTCAGCATCCAACATCTTAGG

Table S1. Mouse-specific primer sequences used for real-time qRT-PCR analyses in the differentiation experiment with US stimulation.

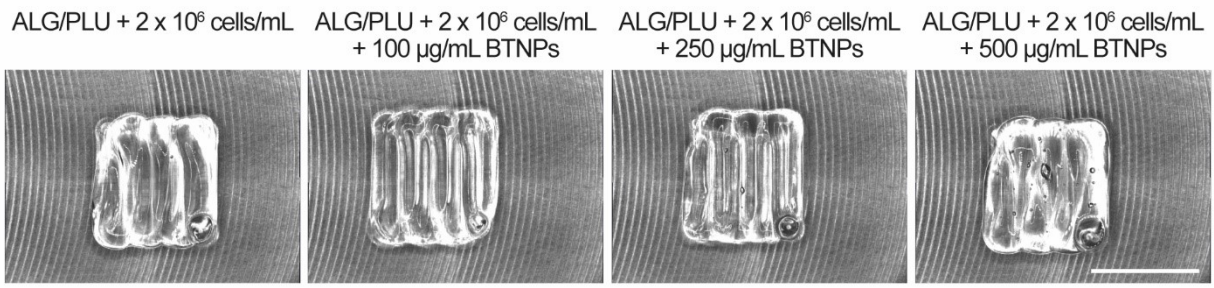

Figure S1. Images of the printed structures composed of one layer of parallelly aligned filaments. Printed structures made of the bare ALG/PLU-based bioink and the doped ones (with 100, 250, and 500 µg/mL BTNPs) are shown. Scale bar: 5 mm. ALG/PLU: alginate/Pluronic-based bioink. BTNPs: barium titanate nanoparticles.

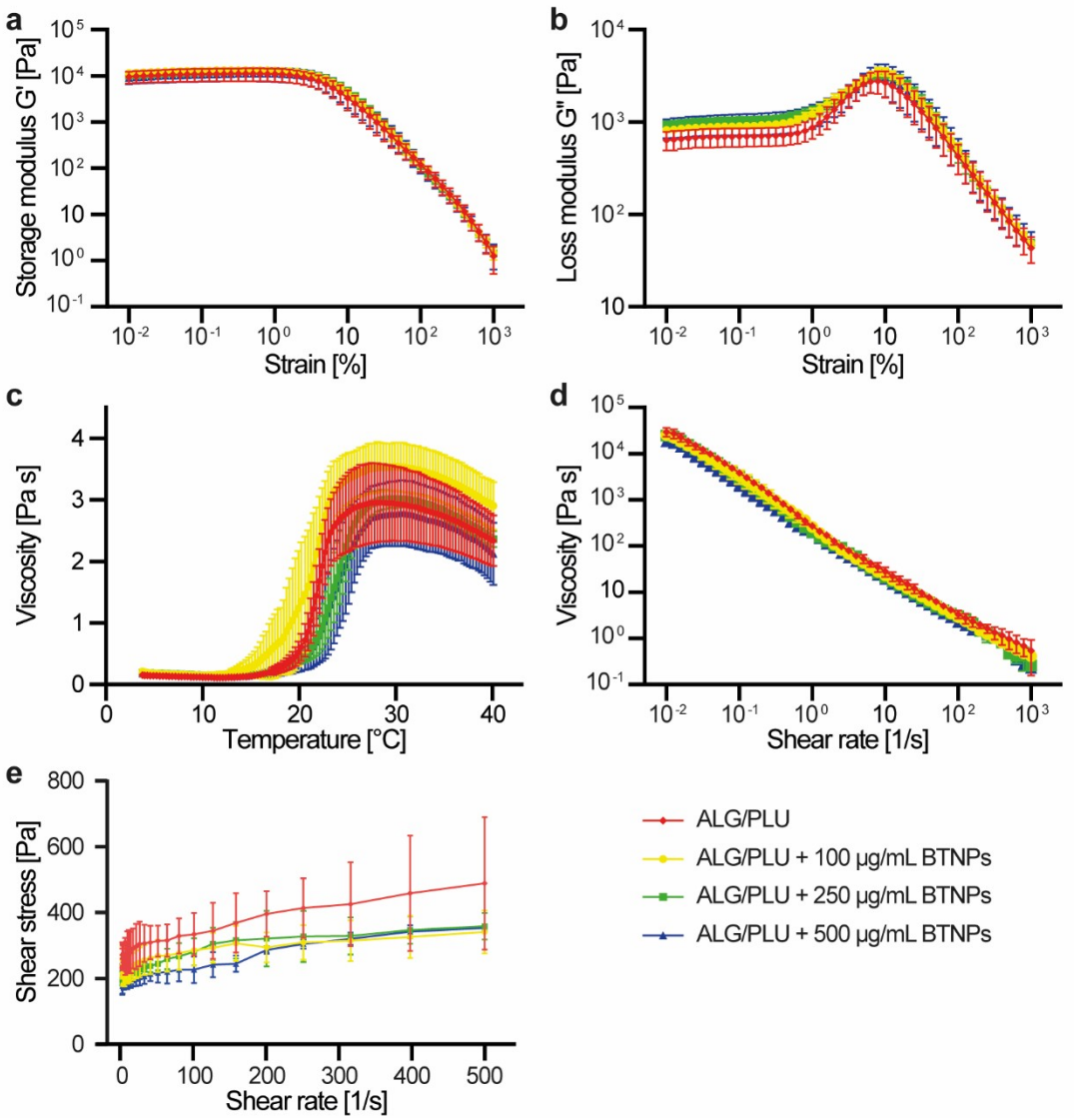

Figure S2. Rheological results obtained with (a,b) strain sweep, (c) temperature sweep, and (d,e) shear rate sweep tests. The graphs illustrate mean values ± standard deviations. ALG/PLU: alginate/Pluronic-based bioink. BTNPs: barium titanate nanoparticles.

Section S1

A preliminary differentiation experiment was carried out to identify the most suitable differentiation medium. In this experiment, the non-doped ALG/PLU-based bioink and the bioink doped with 250 μg/mL BTNPs were loaded with 2×10^6 cells/mL and printed by depositing one single layer (7×7 mm^2) in a 24-well plate, using the printing and crosslinking parameters reported in section 2.5. After structure printing and crosslinking, the printed structures were maintained in growth medium for 6 days. Then, both types of printed structures were divided into two groups that were cultured for 7 additional days into two different differentiation media, namely differentiation medium 1 (DM1) and differentiation medium 2 (DM2). DM1 consisted of DMEM supplemented with 1% FBS, 1% P/S, and 1% ITS. DM2 consisted of DMEM supplemented with 10% horse serum (HS, ECS0091L, Euroclone), 1% P/S, and 50 ng/mL insulin-like growth factor (IGF-1, 250-19, PeproTech).[1–3] The experiment timeline is depicted in Fig. S3a.

At the end-point (day 7 of differentiation), fluorescence imaging and real-time qRT-PCR were performed. For fluorescence staining, the printed structures were gently washed with 1 mL of 0.9% w/v NaCl solution (746398, Sigma-Aldrich). Then, they were fixed in 500 μL of 4% v/v paraformaldehyde (28908, Thermo Scientific) diluted in a 25 mM $CaCl_2$ and 0.9% w/v NaCl solution (15 min at room temperature). After another gentle wash with 25 mM $CaCl_2$ and 0.9% w/v NaCl solution, the printed structures were incubated in 500 μL of 0.1% v/v Triton X-100 (T8787, Sigma-Aldrich) in 25 mM $CaCl_2$ and 0.9% w/v NaCl solution to permeabilize the cells (5 min at room temperature). The staining solution was prepared by diluting tetramethylrhodamine (TRITC)-conjugated phalloidin (P1951, Sigma-Aldrich) (1:400) and Hoechst 33342 (H1399, Invitrogen) (1:1000) in 25 mM $CaCl_2$ and 0.9% w/v NaCl solution. All samples were incubated in the staining solution for 80 min at room temperature. Finally, fluorescence images were acquired using a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany). Representative images were selected for each experimental group.

For real-time qRT-PCR, the protocol described in section 2.9 was performed. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), myogenic differentiation 1 (*MYOD1*), myogenin (*MYOG*), actin alpha 1, skeletal muscle (*ACTA1*), cysteine and glycine-rich protein 3 (*CSRP3*), myosin heavy chain 2 (*MYH2*), myosin heavy chain 4 (*MYH4*), and actinin alpha 2 (*ACTN2*) genes were analyzed with real-time qRT-PCR, using the primers listed in Table S2.

Gene	Primer sequence (5' – 3')
GAPDH	Fw: CGACTTCAACAGCAACTCCCACTCTTC Rev: TGGGTGGTCCAGGGTTTCTTACTCCTT
MYOD1	Fw: TCTCCACAGACAGCCCCGC Rev: GGTTCCCTGTTCTGTGTCGCTT
MYOG	Fw: CCCATGGTGCCCAGTGAA Rev: GCAGATTGTGGGCGTCTGTA
ACTA1	Fw: ATGGTAGGTATGGGTCAG Rev: GATCTTCTCCATGTCGTC
CSRP3	Fw: TGGGTTTGGAGGGCTTAC Rev: CACTGCTGTTGACTGATAGG
MYH2	Fw: GCAGAGACCGAGAAGGAG Rev: CTTTCAAGAGGGACACCATC
MYH4	Fw: GAAGGAGGGCATTGATTGG Rev: TGAAGGAGGTGTCTGTCG
ACTN2	Fw: TCATCCTCCGCTTCGCCATTC Rev: CTTCAGCATCCAACATCTTAGG

Table S2. Mouse-specific primer sequences used for real-time qRT-PCR analyses used in the preliminary differentiation experiment for selecting the differentiation medium.

The results of this preliminary differentiation experiment are shown in Fig. S3b and S3c.

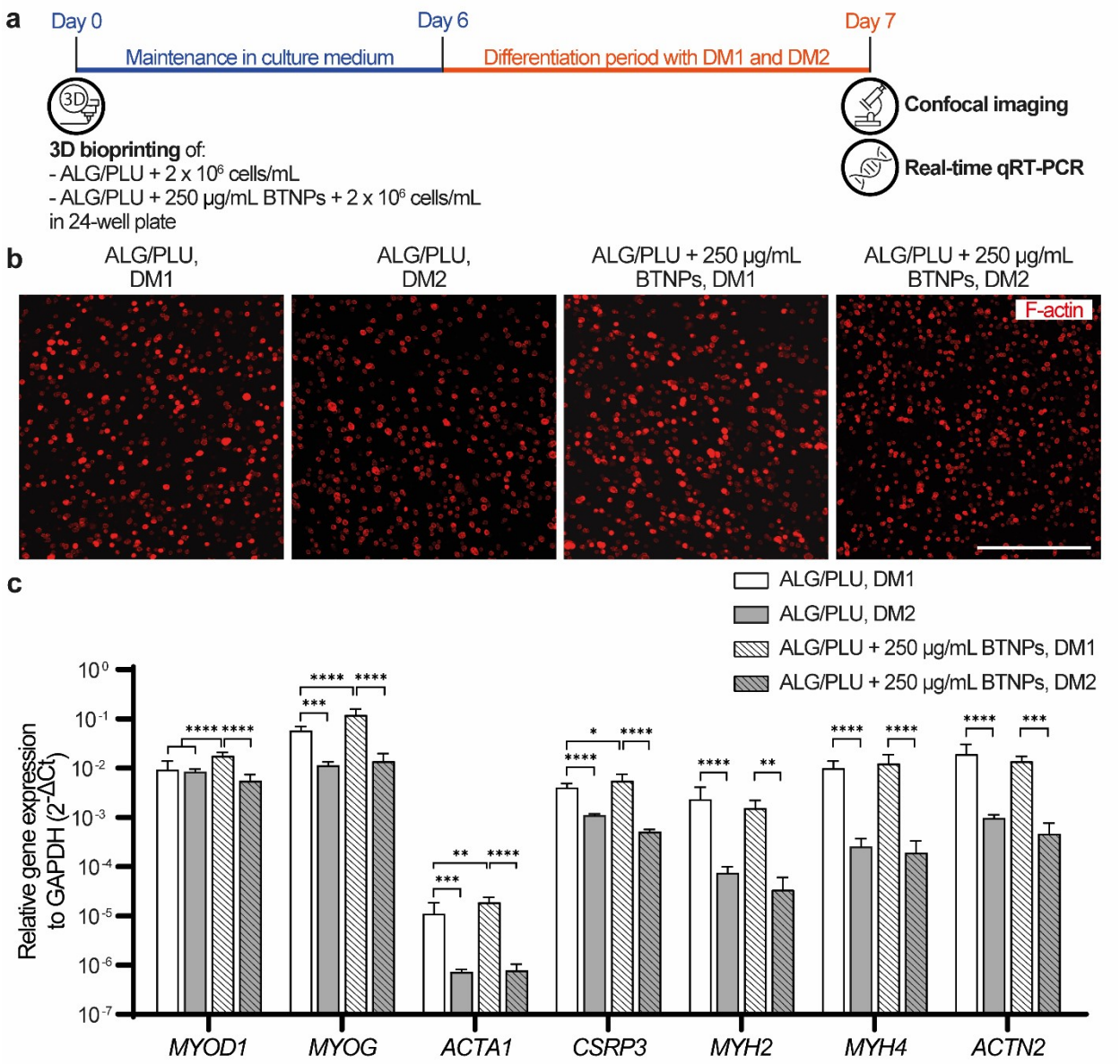

Figure S3. (a) Timeline of the preliminary differentiation experiment. (b) Representative fluorescence images (maximum intensity projections) of differentiating myoblasts embedded in the printed structures (non-doped and doped with 250 µg/mL BTNPs) at the end-point of the preliminary differentiation experiment (day 7 of differentiation). Red: F-actin. Scale bar: 500 µm. (c) Results of the gene expression analysis carried out through real-time qRT-PCR. The graphs illustrate mean values ± standard deviations. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ALG/PLU: alginate/Pluronic-based bioink. BTNPs: barium titanate nanoparticles. DM1: differentiation medium 1. DM2: differentiation medium 2.

Fluorescent staining of the cell actin microfilaments highlighted no evident qualitative differences in terms of cell number, distribution, and shape between the different bioinks composition and differentiation media (Fig. S3b). In fact, cells appeared well distributed in the printed structures of each experimental group, but they were separated from each other and no multinucleated structures were observed. Besides, those images suggested the necessity to increase the cell density to promote cell aggregation, which is crucial for the myogenic process.[4] Thus, for the subsequent differentiation experiment including LIPUS stimulation (described in section 2.9), the cell density was increased.

Gene expression analyses of myogenic markers were performed after 7 days of differentiation, and pointed out the greater myogenic potential of DM1 compared to DM2 (Fig. S3c). For this reason, DM2 was excluded for further differentiation tests. No significant differences were observed between printed

structures non-doped or doped with BTNPs in terms of gene expression of *MYH2*, *MYH4*, *ACTN2,* which are genes expressed at late differentiation stages. *MYOD1*, *MYOG*, *ACTA1*, *CSRP3* resulted over-expressed in the presence of BTNPs. These genes feature the early myogenesis phases.[5] Thus, these preliminary results demonstrated the potential of piezoelectric bioinks to boost myogenesis in 3D bioprinted skeletal muscle constructs.

References

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