Supporting Information

Multi-photon polymerization of bio-inspired, thymol-functionalized hybrid materials with biocompatible and antimicrobial activity

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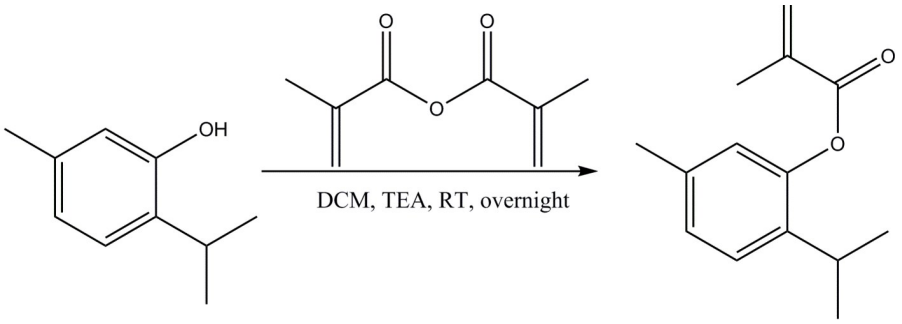
Materials

All solvents were purchased from Sigma-Aldrich (Steinheim, Germany) and were used without further purification. Thymol (98.5%), methacrylic anhydride (94%), methacryloxypropyl trimethoxysilane (MAPTMS, 97%), 2-(dimethylamino)ethyl methacrylate (DMAEMA, 98%), zirconium n-propoxide (ZPO, 70% in propanol), 4,4-bis(diethylamino) benzophenone (BIS, >99%), triethylamine (TEA, 99%), ascorbic acid, phalloidin-Atto 488, 4',6-diamidino-2-phenylindole (DAPI) and Alizarin Red S were purchased from Sigma-Aldrich (Steinheim, Germany). Magnesium sulfate anhydrous (>98%) and HCl (1 M solution) were obtained from Fluka. Silica gel was purchased from Alfa Aesar. Cell culture medium, (alpha-MEM), fetal bovine serum (FBS), antibiotic/antimycotic, glutamine and the PrestoBlue® reagent were purchased from Invitrogen (Karlsruhe, Germany) and Triton X-100 from Merck (Darmstadt, Germany).

Thymyl methacrylate (THYMA) synthesis

Thymol (3.5 g, 23.3 mmol), dichloromethane (DCM) (10 mL) and triethylamine (TEA) (6.49 mL, 46.6 mmol) were introduced in a dried round bottom flask. Then, methacrylic anhydride (3.82 mL, 25.6 mmol) was added and the reaction mixture was stirred at 40°C, overnight. After 24 h, the triethylammonium salt formed was removed by filtration and was washed with diethyl ether. The filtrate was extracted with a concentrated $NaHCO_3$ solution. The organic phase was dried with anhydrous magnesium sulfate, was filtered and the solvent was removed in a rotary evaporator to obtain a yellow viscous liquid. The product was purified by column chromatography using petroleum ether/ethyl acetate (98/2 v/v %) as the mobile phase. The fractions were finally evaporated to remove the solvent and afford the product as a clear syrup (3.56g, 70% yield).



Scheme S1: Schematic representation of the synthetic procedure followed for the synthesis of THYMA

^{1}H / ^{13}C NMR spectroscopy

^{1}H/^{13}C NMR spectra of THYMA were obtained on a Bruker AMX-500 spectrometer (Billerica, MA, USA) in deuterated dichloromethane.

NMR analysis:

^{1}H NMR (500MHz, CD_2Cl_2): δ 7.27-7.28 (d), δ 7.09-7.11 (m, 1H), δ 6,9 (s, 1H), δ 6.39 (m, 1H), δ 5.8 (m, 1H), δ 3.01-3.08 (hept), δ 2.37 (s, 3H), δ 2.12 (m, 3H), δ 1.24 (s, 3H).

^{13}C NMR (500 MHz, CD_2Cl_2): δ 165.82, 148.05, 136.99, 136.46, 135.85, 126.68, 126.49, 126.12, 122.56 26.97, 22.49, 20.28, 17.97.

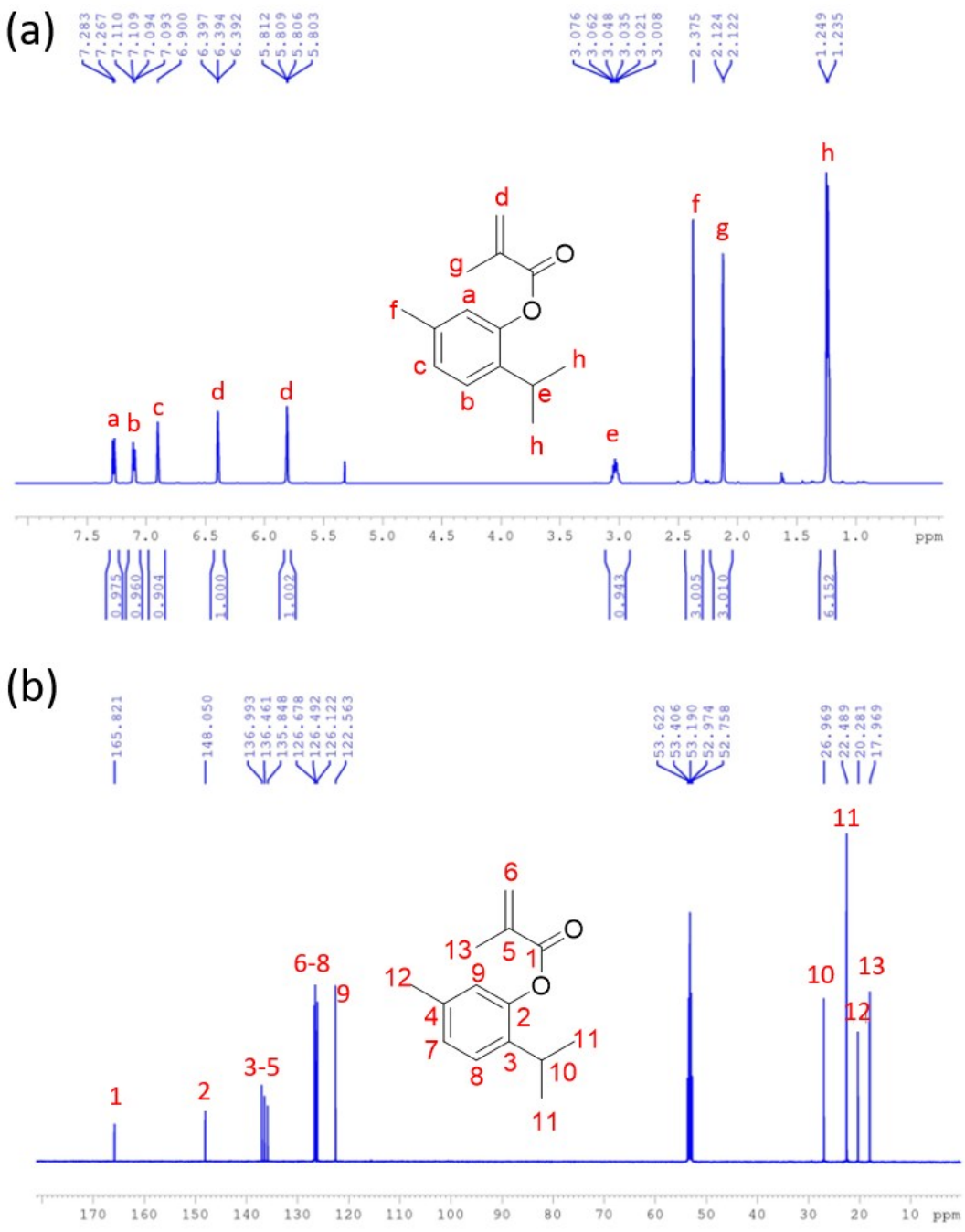


Figure S1: (a) ^{1}H NMR and (b) ^{13}C NMR spectra of THYMA in CD_2Cl_2

FTIR spectroscopy

Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectra were recorded on a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For each spectrum, 128 scans were collected in the 400–4000 cm^{-1} range.

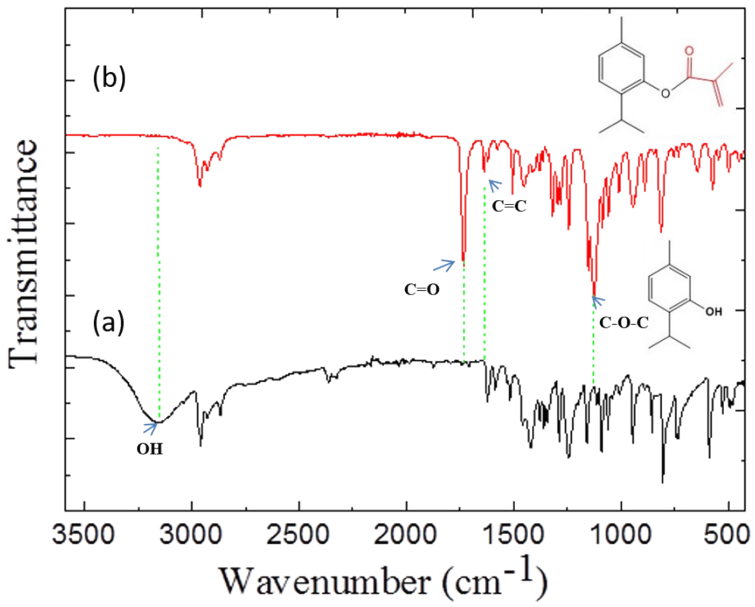


Figure S2: FTIR spectra of (a) thymol and (b) THYMA

THYMA-hybrid material synthesis

MAPTMS (1 ml, 4.2 mmol) was hydrolyzed using 0.01 M HCl (1:0.1 v/v MAPTMS:HCl). In a separate flask, DMAEMA (0.47 ml, 2.8 mmol) was mixed with ZPO (0.87 ml, 2.8 mmol). After stirring for 15 min, the DMAEMA/ZPO mixture was added dropwise into the solution of hydrolyzed MAPTMS and the mixture was stirred for another 15 min. Next, THYMA (0.6 g, 2.8 mmol) was added, and the mixture was left under stirring overnight. Finally, BIS (0.02 g, 6.2 x 10^{-5} mol, 1 wt% to the monomers) was added, and the solution was stirred for another 15 min. The mixture was filtered with a 0.45 µm pore size filter to remove any undissolved photoinitiator.

Hybrid material synthesis

The hybrid material used as a control sample was prepared by hydrolysing MAPTMS (1 ml, 4.2 mmol) with 0.01 M HCl (1:0.1 v/v MAPTMS:HCl). In a separate flask, DMAEMA (0.47 ml, 2.8 mmol) and ZPO (0.87 ml, 2.8 mmol) were mixed and were allowed to stir for 15 min. Next, the DMAEMA/ZPO mixture was added dropwise into the hydrolyzed MAPTMS and the mixture was stirred for an additional 15 min. Finally, BIS (0.02 g, 6.2 x 10^{-5} mol, 1 wt% with respect to the monomers) was added, and the solution was stirred again for 15 min. The mixture was filtered with a 0.45 µm pore size filter to remove any undissolved photoinitiator before being deposited onto the glass substrates.

Glass substrate preparation

The glass substrates used to fabricate the polymer films and the 3D structures were cleaned by sonication in acetone for 1 h, followed by rinsing with methanol for 30 min and with deionized water multiple times. To enhance the adhesion of the fabricated 3D structures onto the substrates, the latter were modified with MAPTMS. For this, the substrates were placed in a toluene:MAPTMS solution (1:250 v/v) under sonication for 4 h, followed by rinsing with ethanol several times, and were finally dried under a nitrogen gas stream.

Preparation of thin films of the materials

40 µl of the above-mentioned photosensitive hybrid materials were spin-coated (4000 rpm for 60 s) on 100 nm thick cover glass slips with a 13 mm diameter. After drying and condensation of the alkoxide moieties, the organic vinyl bonds of the material were photopolymerized, using a UV lamp at 365 nm, to form a hybrid double network. Finally, the polymerized films were developed for 1 h in 4-methyl-2-pentanone and dried at 100 °C for 30 min.

Fabrication of 3D scaffolds by MPP

The samples were prepared by drop-casting the hybrid and THYMA-hybrid materials onto 100 µm thick glass substrates functionalized with MAPTMS. After drying and condensation of the alkoxide moieties by placing the samples under vacuum, the material was polymerized by multi-photon polymerization. The experimental setup used for the fabrication of the 3D structures has been described previously. A Ti:Sapphire femtosecond laser beam (Femtolasers Fusion, 800 nm, 75 MHz, 20 fs) was tightly focused into the volume of the photosensitive material using a 20x microscope objective lens with NA of 0.8 (Zeiss, Plan Apochromat).[1] Sample movement in the XY plane was achieved using an x-y galvanometric mirror digital scanner (Scanlabs Hurry-Scan II), while for the z-axis linear stages (Physik Instrumente) were employed. The writing procedure was controlled by a computer using the SAMlight software. The structures were fabricated in a layer-by-layer process with the last layer attached to the glass substrate.

Cell culture

Human dental pulp stem cells (DPSCs) were isolated from extracted third molars of young healthy donors after inform consent, according to an approved protocol by the School of Dentistry, Aristotle University of Thessaloniki and the Institutional Ethics Committee (322/15-04-2013). DPSCs cultures were established using the enzymatic dissociation method as previously described, and immunophenotypically characterized by flow cytometry.[2]

Early passages (2–4) of DPSCs were grown in cell culture flasks using alpha-MEM, supplemented with glutamine (2 mM), penicillin (50 IU/ml), streptomycin (50 g/ml), amphotericin B (0.25 mg/ml), 100 µM ascorbic acid and 15% FBS in a humidified atmosphere and 5% CO_2 at 37 °C in a cell culture incubator (Thermo Scientific). Confluent cells were detached and passaged after trypsination with 0.25 % trypsin- 1mM EDTA, seeded at a 90% confluence, and allowed to grow for 4–5 days before the next passage.

Cell viability and proliferation assay

A suspension of 3 x 10^4 cells in alpha-MEM was seeded on the photopolymerized hybrid and THYMA-hybrid material films and the samples were placed into the cell culture incubator at 37 °C. On days 2, 4, and 7 post-seeding, the cell viability and proliferation assay was performed using the resazurin-based PrestoBlue® reagent according to the manufacturer's instructions. The reagent was incubated on the cells at 37 °C for 60 min. The absorbance was measured by a spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA) and the cell number quantification was performed by means of a calibration curve. Error bars,

representing the average of triplicates ± standard deviation in two independent experiments, were calculated (n=6).

Optical microscopy

A suspension of 2 x 10^4 cells in alpha-MEM was seeded on glass substrates covered either with films of the photopolymerized hybrid materials or with nine two-photon polymerized 3D scaffolds of the materials with dimensions 555 x 555 x 50 μm^3. The specimens were placed in the cell culture incubator at 37 °C and the cells on them were examined daily for 7 days and visualized by optical microscopy by means of a Zeiss Axiovert 200 microscope. Images were taken by a ProgResVR CFscan Jenoptik camera (Jena, Germany) using the ProgResVR CapturePro 2.0 software and objective lenses with 10-fold magnification.

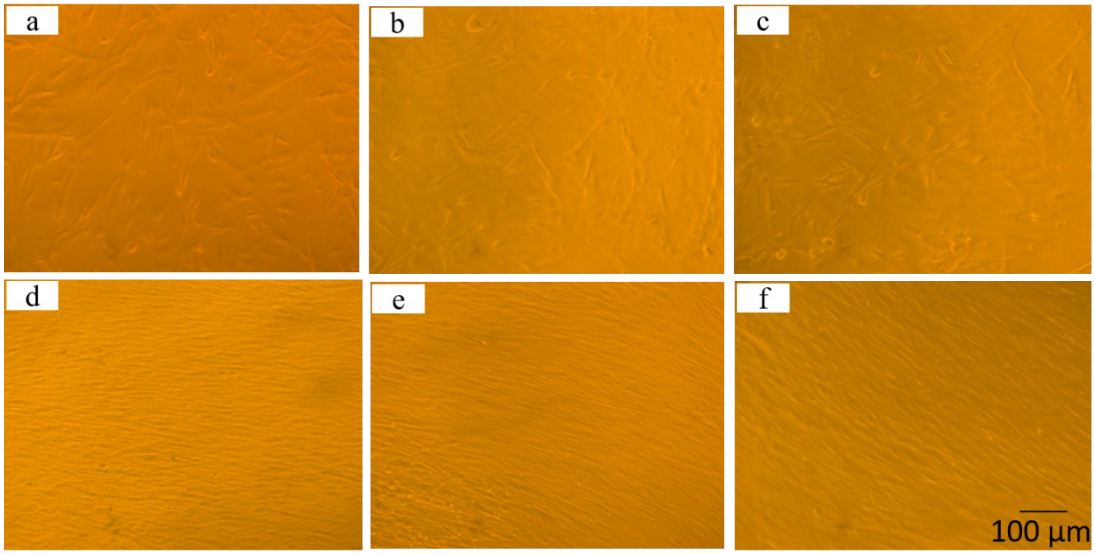


Figure S3: Optical microscopy images showing the adhesion of human dental pulp stem cells on the hybrid material films (a,d), the THYMA-hybrid material films (b,e), and tissue culture treated polystyrene (TCPS) control (c,f) after 2 days (a,b,c upper panel) and 7 days (d,e,f lower panel) in culture. The scale bar represents 100 µm and is the same for all images.

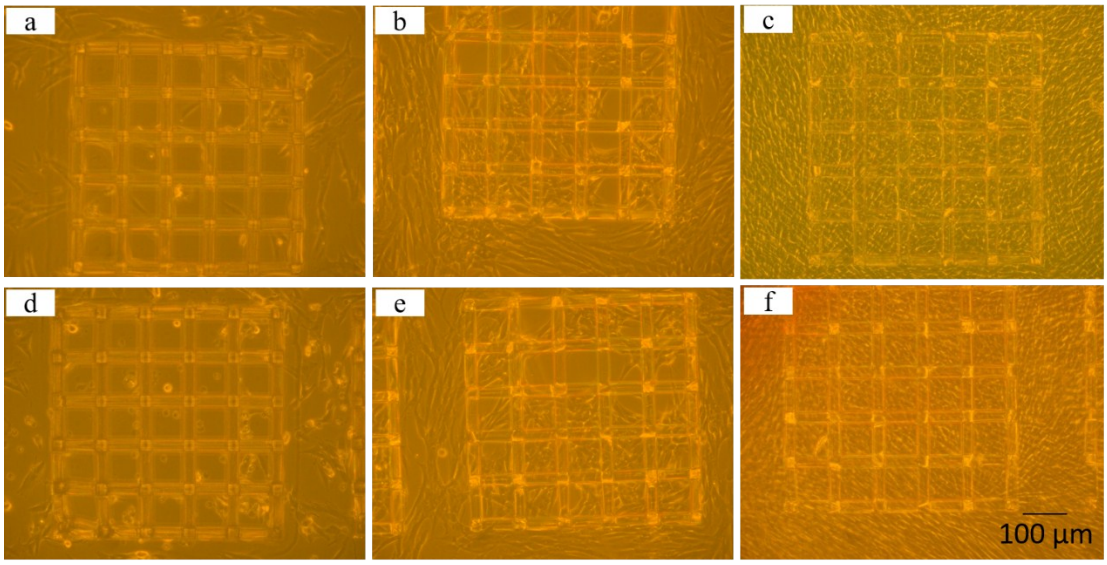


Figure S4: Optical microscopy images showing the adhesion of human dental pulp stem cells onto multi-photon polymerized grid-shaped 3D scaffolds after 2 days (a,d), 4 days (b,e) and 7 days (c,f) in culture. The upper panel images (a,b,c) represent the hybrid material and the lower panel (d,e,f) the THYMA hybrid material cultures. The scale bar represents 100 µm and was the same for all images.

Scanning electron microscopy

A suspension of 2 x 10^4 cells in alpha-MEM was seeded on the hybrid and THYMA-hybrid material 3D scaffolds and the samples were placed in the cell culture incubator at 37 °C for up to 7 days. Specimens were then removed from the incubator and rinsed three times with PBS, fixed with 4% paraformaldehyde for 30 min, and dehydrated in increasing concentrations (from 30 to 100%) of ethanol. The specimens were next dried in a critical point drier (Baltec CPD 030), sputter-coated with a 10 nm thick layer of gold and observed under a scanning electron microscope at an accelerating voltage of 20 kV.

Laser scanning confocal microscopy

A suspension of 2 x 10^4 cells in alpha-MEM was seeded on the hybrid and THYMA-hybrid material 3D scaffolds and the samples were placed in the cell culture incubator at 37 °C for up to 7 days. After the incubation time, the samples were rinsed with PBS, fixed with 4 % PFA for 15 min and permeabilized with 0.1 % Triton X-100 in PBS for 5 min. The non-specific binding sites were blocked with a 2 % BSA solution in PBS for 30 min. The actin cytoskeleton was stained by incubating the cells on the samples in 20 µl diluted phalloidin-Atto 488 blocking solution for 1 h at 37 °C and were subsequently stained by simultaneous incubation in 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Finally, the samples were washed with PBS, mounted using a mounting fluid and observed under a LEICA TCS SP8 laser scanning confocal microscope.

Functionalization of the scaffolds with rhBMP-2

Recombinant human bone morphogenetic protein-2 (rhBMP-2) (Prospecbio International, Ness-Ziona, Israel) was physically adsorbed onto the MPP 3D scaffolds according to a previously described protocol.[3] Briefly, the samples containing the photopolymerized hybrid and THYMA-hybrid material scaffolds were incubated overnight in a 0.1 mg/ml rhBMP-2 solution and were rinsed with sodium acetate buffer and cell culture medium prior to seeding cells on them as described above in the cell viability and proliferation assay section.

Alizarin red staining

The cell viability, expressed as number of living cells, was measured using the PrestoBlue® assay. The samples were washed with PBS, fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min and again washed three times with PBS. Next, the samples were stained with a 2 % solution of the dye (Alizarin Red S) in water. The pH of the solution was close to 3.7, and was adjusted between 4.1 and 4.3 by the addition of ammonia. Then, 500 µl of the dye solution were added to each well and the samples were stained for 45 min. The excess dye was removed and the samples were washed thoroughly with deionized water. A 10 % cetylpyridinium chloride (CPC) solution in 10 mM Na_2PO_4, pH 7.0, was prepared. Before extraction with CPC, the cell layer was removed from the glass substrate, surrounding a block of three two-photon polymerized structures, by means of a cell scraper. In this way, only the stained cells residing within three continuous grid-shaped scaffolds were included in the quantification of the Alizarin Red extraction. Alizarin red was extracted from the monolayer by incubation of the samples in 100 µl CPC in buffer for 1 h at room temperature under shaking. The dye was finally removed and 50 µl aliquots were transferred to a 96-well plate prior to reading the absorbance by a spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek, Bad Friedrichshall, Germany) at 550 nm. The OD values from the alizarin staining were normalized to the number of living cells, determined by the cell viability assay prior to the alizarin staining.

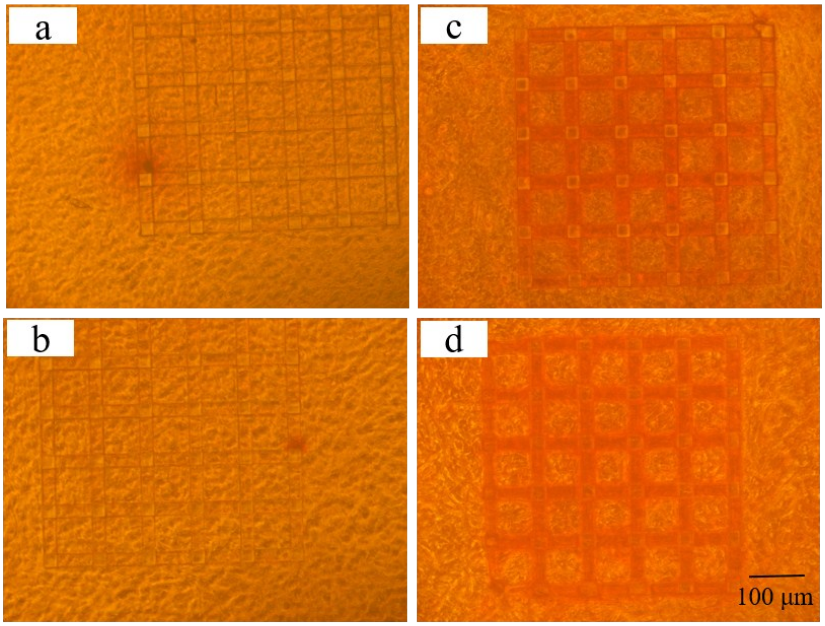


Figure S5: Optical microscopy images showing grid-shaped hybrid (a) and THYMA-hybrid 3D scaffolds (b), and BMP-2-functionalized hybrid (c) and THYMA-hybrid (d) 3D scaffolds seeded with human dental pulp stem cells for 21 days and stained with Alizarin Red S. The scale bar represents 100 µm in all images.

Statistical analysis

Statistical analysis was performed using the one-way ANOVA Dunnett's multiple comparisons test. To statistically evaluate the difference in cell proliferation at certain time points (2, 4, and 7 days), we compared the hybrid material films at each time-point against the control tissue culture polystyrene (TCPS) surface. Statistical significance was set at $p < 0.05$.

Antibacterial assays

The antimicrobial activity of the 3D scaffolds was investigated using *B. cereus* (ATCC 14579) and *E. coli* as model gram-positive and gram-negative microorganisms, respectively. *B. cereus* and *E. coli* were cultivated by streaking

on Luria-Bertani (LB) agar plates and were incubated at 37 °C for 24 h. Then individual colonies were picked and incubated in LB liquid culture medium. Both strain cultures were shaken at 37 °C for 24 h, and then diluted with LB to obtain a predetermined bacteria concentration of 1×10^8 CFU/ml. The 3D scaffolds were rinsed with methanol and sterilized at 120 °C for 30 min, prior to the antibacterial testing procedure. Next, the samples were placed in a 12-well plate, 1 ml of the *B. cereus* or *E. coli* suspension was pipetted into each well and were incubated at 37 °C for 4 days. Every day, fresh LB culture medium was added to the samples to promote bacterial growth and biofilm formation. Samples at day 1 and day 4 in culture were washed gently with sterile PBS and were next immersed in 1 ml fresh PBS at a shaking speed of 150 rpm for 1 h, to remove the non-attached bacteria. Next, the samples were fixed with 2% paraformaldehyde and 2% glutaraldehyde in sodium carboxylate buffer (SCB) at 4 °C for 20 min, were rinsed twice with SCB to remove the unreacted fixatives, and were dehydrated in a series of washes with increasing ethanol concentration from 30 to 100%. Finally, the samples were dried in a critical point dryer (BalTec CPD 030), were sputter-coated (BalTec SCD 050) with a 10 nm thick layer of a Pd-Au alloy and were imaged using a scanning electron microscope (SEM, JEOL JSM-840) at an accelerating voltage of 20 kV to visualize the bacterial attachment.

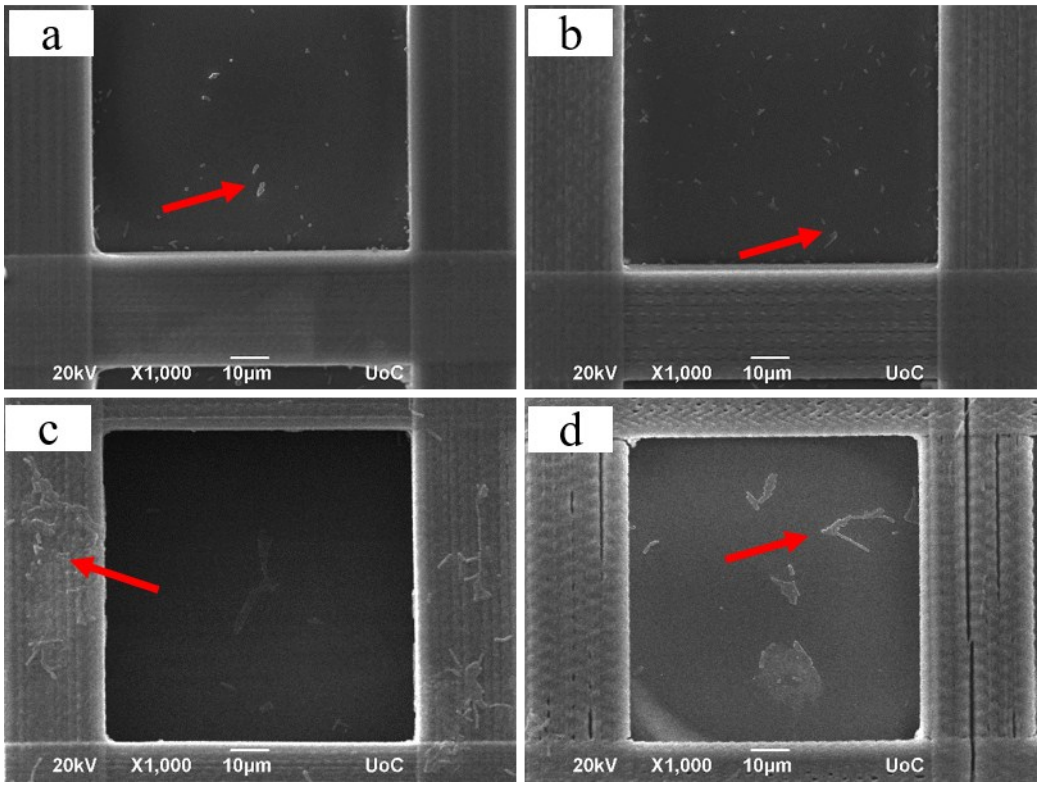


Figure S6: SEM images of *E. coli* (a,b) and *B. cereus* (c,d) after 1 day in culture on the hybrid (a, c) and THYMA-hybrid (b,d) material 3D scaffolds. Red arrows indicate bacteria attached on the glass substrate or on the 3D scaffolds.

Water contact angle measurements

Wettability was assessed by static water contact angle (WCA) measurements using a contact angle goniometer (OCA-40, Dataphysics) and the sessile drop method. A 5 μL droplet of nanopure water was used and the contact angles were calculated from the digital images of the water droplets deposited on the surfaces, recorded by a camera, using the appropriate software.

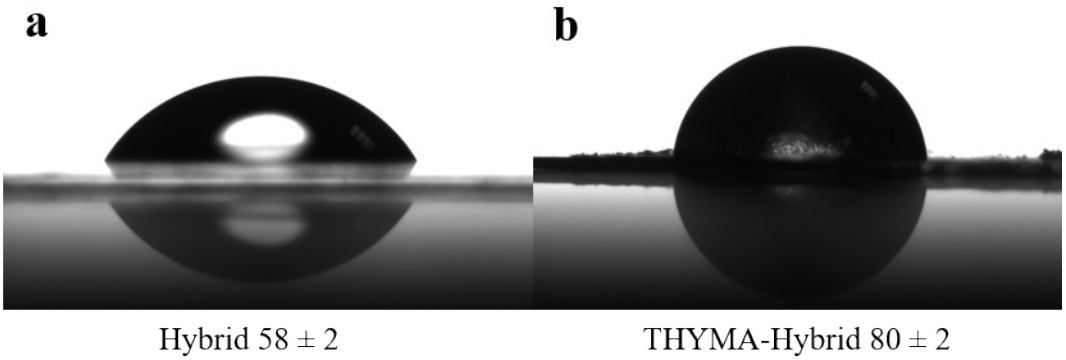


Figure S7: Static water contact angles on 2D photopolymerized films of the (a) hybrid and (b) THYMA-hybrid materials.

1. M. Farsari, M. Vamvakaki and B. N. Chichkov, *J. Opt.*, 2010, **12**,124001
2. A. Bakopoulou, A. Georgopoulou, I. Grivas, C. Bekiari, O. Prymak, K. Loza, M. Epple, G. C. Papadopoulos, P. Koidis and M. Chatzinikolaidou, *Dent. Mater.*, 2019, **35**, 310-327.
3. M. Chatzinikolaidou, C. Pontikoglou, K. Terzaki, M. Kaliva, A. Kalyva, E. Papadaki, M. Vamvakaki and M. Farsari, *Colloids Surf., B* 2017, **149**, 233-242.