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Osteoconductive biocompatible 3D-printed composites of poly-D,L-lactide filled with nanocrystalline cellulose modified by poly(glutamic acid)

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A. Mechanical Tests

Mechanical tests were carried out using Shimadzu AG-100X Plus machine (Kyoto, Japan) at room temperature in uniaxial compression mode with a compression speed of 2 mm/min. Hot compression molding was used to prepare specimens for study of the mechanical properties. A monolithic cylindric specimens were prepared by hot molding at 190 °C and were of the following dimensions: 10 mm in diameter and 3 mm in height. Three specimens of each composition were tested. The stress-strain curves obtained for pure poly(D,L-lactic acid) (PDDLA) and its composites are shown in **Figure S1**.

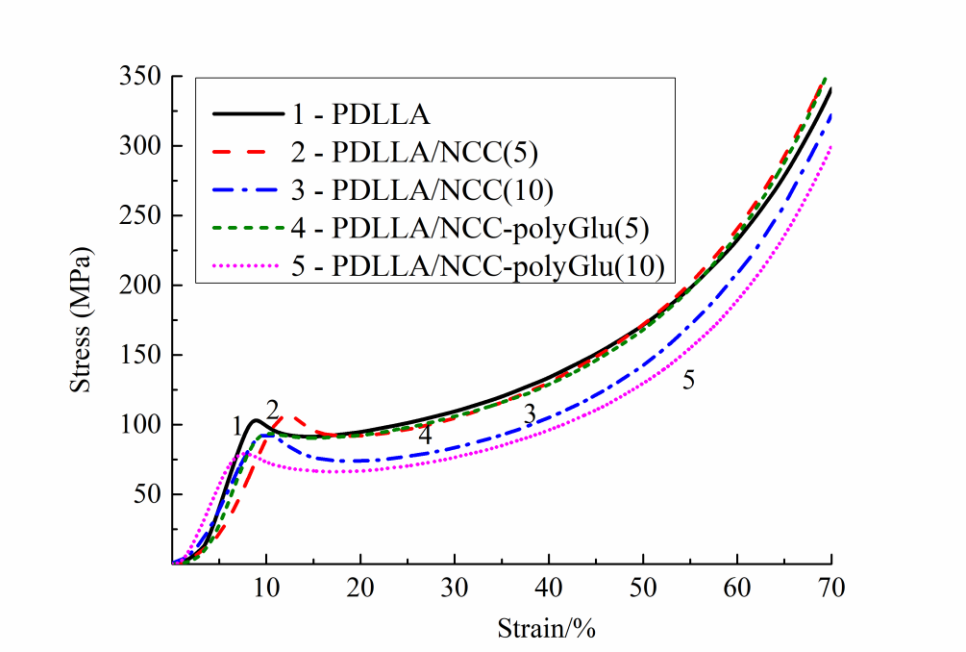

Figure S1. The stress-strain curves of PDLLA and its composites with NCC and polyGlu-NCC evaluated in compression tests.

Mechanical characteristics were calculated according to the standard method: by dividing the maximum force, recorded at the moment of stopping the experiment, to the initial section of the sample before testing. The experiment was carried out up to the 70 % deformation.

B. 3D printing of Polymer Scaffolds

GeSim 3D printer with pneumatic extruder (Radeberg, Germany) was utilized to produce the 3D printed matrices with interpenetrating pores using FDM technology. PDLLA was loaded into a cartridge of the extruder with a diameter of printing head of 0.3 mm. A temperature of 180 °C was not enough to extrude the material, while at 190 °C there was its uncontrolled spontaneous outflow from the extruder. In this regard, the temperature of the cartridge and head was set to 185 °C. Preheating for 30 min before printing was required to obtain reproducible material. A feature of the mechanical extruder operation is that the uniform movement of the plunger at start is preceded by a given distance (up to 3 mm), which affects the uniformity of further printing. To optimize the starting conditions, the distance settings were gradually reduced from 3.0 mm to 0.2 mm, and the speed was reduced from 3 mm/s to 1 mm/s to ensure uniformity in the process. For the optimization of further printing settings, the plunger speed was varied between 1 and 20 μm/s in steps of 5 μm/s. The optimal speed providing continuous uniform extrusion was 5 μm/s. A step-by-step description of 3D printing optimization, supported by images of printed materials, is provided below.

Printing of specimen 1 (**Figure S2**, **specimen 1**) was started without heating the substrate (at room temperature 22°C). The initial printing parameters were set as follows: plunger speed – 5 μm/s, cartridge temperature – 185 °C, layer height – 0.29 mm, print head speed – 1.0 mm/s, distance from the print head to the printed layer – 0.75 mm. Unfortunately, the threads adhere poor to the glass support and did not form a layer with a constant height. To overcome this drawback, the temperature of the glass support was raised to 70 °C. However, sample 2 was still not completely in contact with the glass (**Figure S2**, **specimen 2**). A successive decrease in the distance from the print head to the printed layer from 0.75 mm to 0.55 mm (**Figure S2**, **specimen 3**) and to 0.35 mm (**Figure S2**, **specimen 4)** led to a significant improvement in the adhesion of the polymer melt and the formation of a flattened first layer. The "tear-off" movement of the printhead from the sample was set in the horizontal direction for all samples. The latter was due to the fact that the last straight section of the track was detached from the support, making it difficult to print subsequent layers in the vertical or diagonal direction.

In addition, to improve the uniformity at the beginning of the first layer, a pause (0.2 s) in the movement of the printhead at the start of layer printing was added. This resulted in the formation of a polymer drop at the layer inception (**Figure S2, specimen 5**). A polymer thread with a constant width in straight sections was created by reducing the speed of the printhead from 1.0 to 0.8 mm/s (**Figure S2, specimen 6**). An attempt to print a multi-layer object using the parameters defined above resulted in layers with irregularly shaped threads (**Figure S2, specimen**

7). Adding of 30 s pause between printing the layers for specimen 8 made it possible to significantly improve the result by removing the effect of excessive fusion of layers with each other (**Figure S2**, **specimen 8**). For specimens 7 and 8, there was insufficient material extrusion for all layers except the first. Increasing the speed of the extruder plunger for layers, starting from the second, from 5 μm/s to 7 μm/s and 10 μm/s (**Figure S2**, **specimens 9** and **10**, respectively) resulted in a product with a constant width of the polymer threads, providing a three-dimensional porous structure.

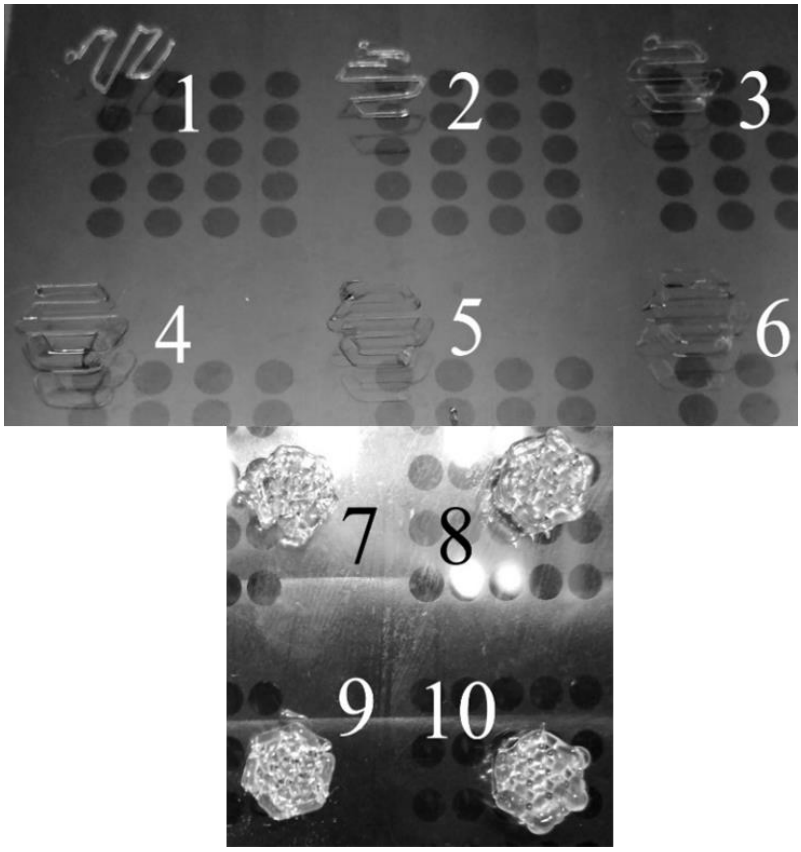

Figure S2. Images of 3D-printed PDLLA samples. 3D printing condition development.

Developed optimal parameters for 3D printing: cartridge and head temperature: 185°C; the speed of the uniform movement of the plunger for the first layer: 5 μm/s, and for subsequent: 10 μm/s; "starting step" of the plunger: 0.2 mm, speed: 1 mm/s; horizontal movement of the printhead "separation" from the product; print head speed: 0.8 mm/s; layer height: 0.29 mm; distance from the print head to the printed layer: 0.35 mm; pause between successive layers: at least 30 seconds; support temperature: 70°C. The laying of subsequent layers was carried out with the rotation of each subsequent plane relative to the previous one by 60º. The PDLLA composite specimens containing 5 and 10 wt% of NCC or polyGlu-modified NCC were 3D-printed using the developed optimal settings.

C. Biological Experiments

Sterilization of the 3D-printed scaffolds was carried out at 37 °C in 70 % ethanol for 3 h and followed by further washing with phosphate buffered saline (0.01 M PBS, pH 7.4). Each polymer matrix was seeded with adipose derived human mesenchymal stem cells (hMSCs, Cell Lines Service GmbH, Eppelheim, Germany). Cells were cultivated in αMEM (Lonza, Allendale, NJ, USA) supplied with 10% human serum at 37 °C, 10 % O_2, 5 % CO_2 for 2 weeks (growth medium). Afterwards half of the samples were transferred to the osteogenic differentiation medium (αMEM, 2.5% human platelate lysate, 0.1% 0.1 mM dexamethasone, 0.5% gentamicin, 1% 500 mM sodium β-glycerophosphate, 0.1% 200 mM L-ascorbic acid 2-phosphate) for 1 week. The surface of the scaffolds before [**Figure 3(***a*)] and after seeding cells [**Figure 3**(*b*)], and biomineralization [**Figure 3**(c)] was studied using scanning electron microscopy (SEM).

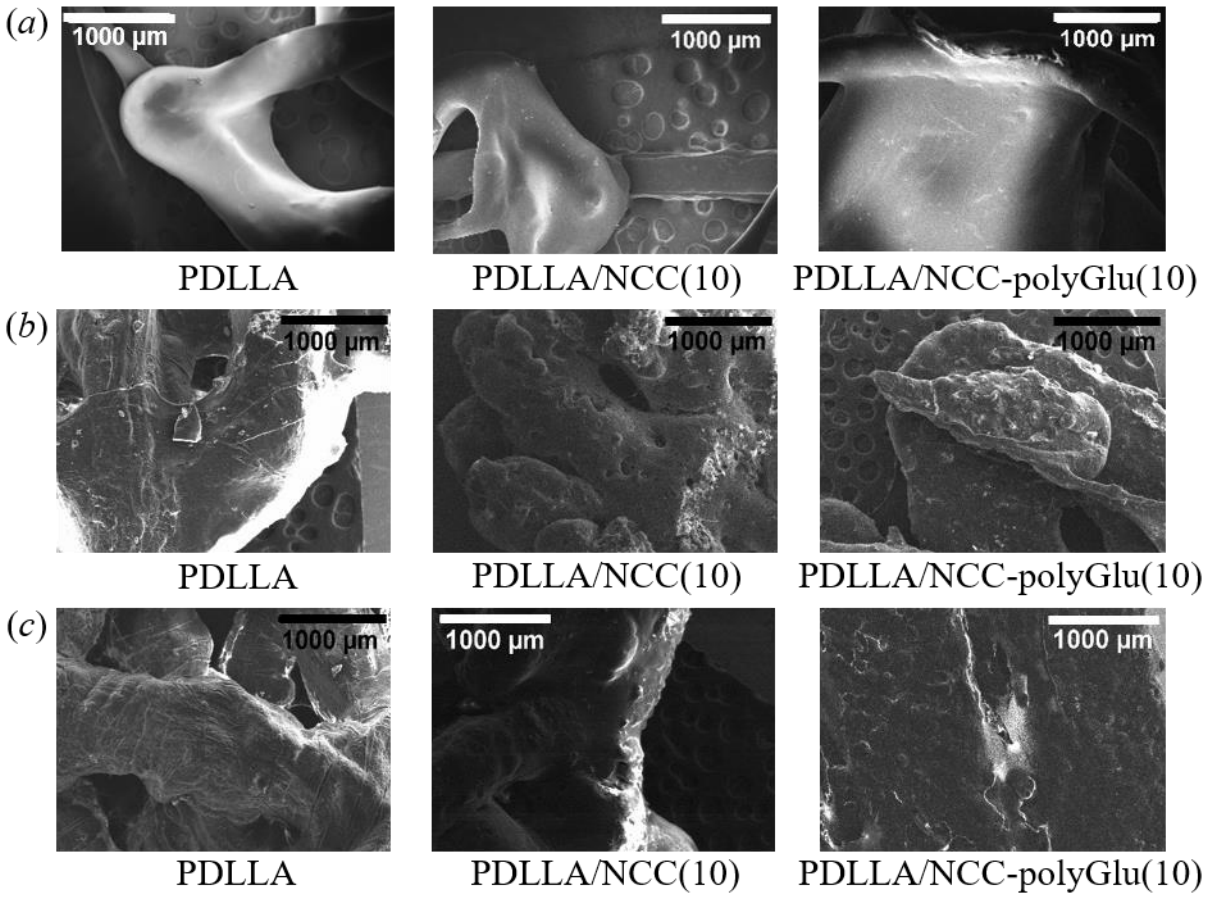

Figure S3. SEM images 3D matrices: (a) before cell experiments, (b) after incubation in growth medium, (c) after osteodifferentiation.

Part of the samples was analyzed by CellTiter-Blue (CTB) viability assay to compare the adhesion and proliferation of the cells at the surface of different scaffolds during their incubation in a growth medium. CTB cell viability assay reagent was purchased from Promega Corp.

(Madison, WI, USA). PDLLA was used as a control, cell adhesion on its surface was taken as 100 %. The results of CTB assay are demonstrated in **Figure S4**.

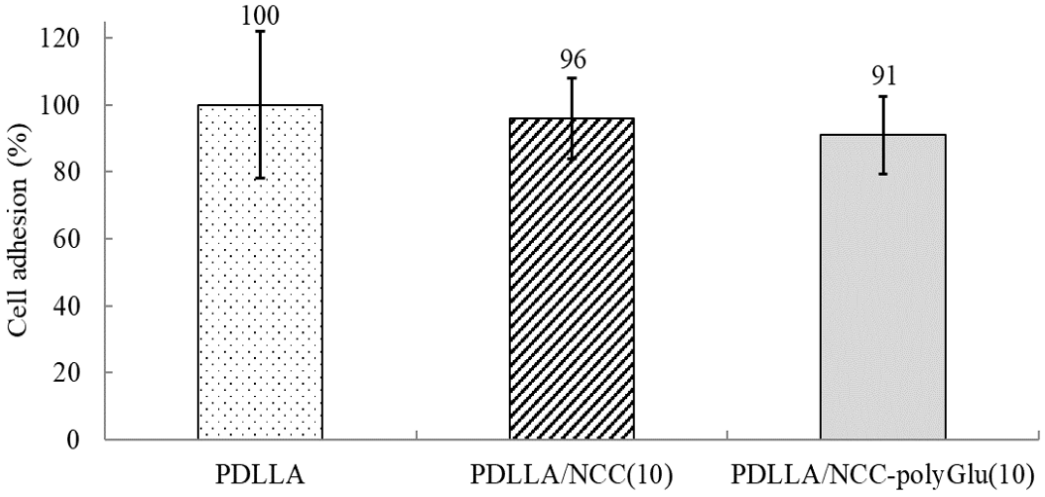

Figure S4. Comparison of the amount of the living cells at the surface of different 3D-printed polymer scaffolds after 2-week incubation in a growth medium.

D. Energy Dispersive X-ray (EDX) Analysis

EDX analysis was performed using Oxford Instruments INCA x-act device and INCA Energy software (High Wycombe, UK). Determination of the relative content of calcium was carried out in total at 10-16 points of the material. Examples of the total spectra for the developed three-dimensional sample containing polyGlu-modified NCC as a filler are shown in **Figure S5**. The quantitative data on Ca-content in at% are given in **Figure S6**.

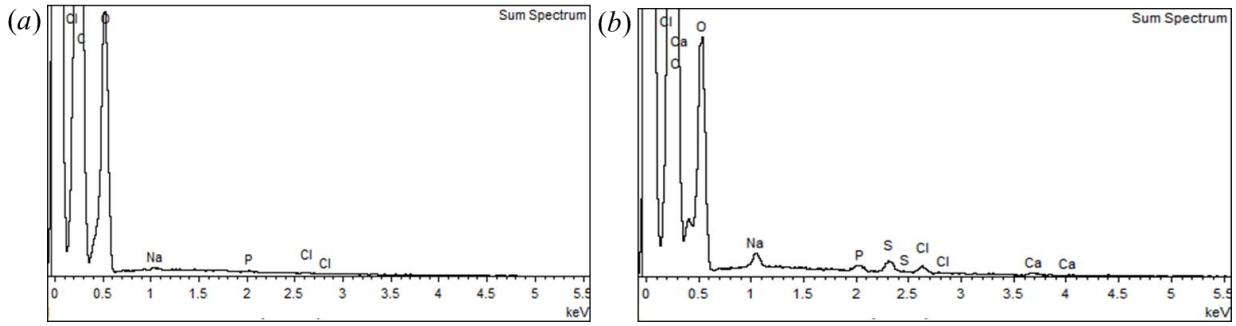

Figure S5. EDX spectra for PDLLA/NCC-polyGlu(10) scaffold after hMSCs growth (a) and after hMSCs osteodifferentiation (b).

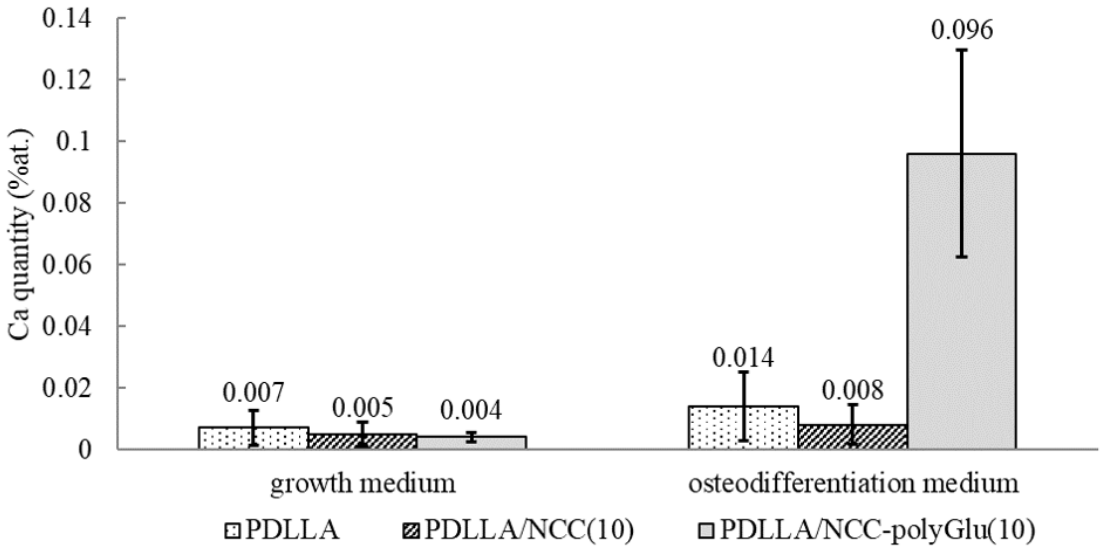

Figure S6. Content of calcium on the surface of various scaffolds (EDX analysis after growth/osteodifferentiation of hMSCs).