Bone-on-a-Chip: A Microscale 3D Biomimetic Model to Study Bone Regeneration

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Organ-on-chip models, developed using microengineering and microfluidic technologies, aim to recreate physiological-like microenvironments of organs or tissues as a tool to study (patho)physiological processes in vitro. On-chip models of bone are relevant for the study of bone physiology, diseases and regenerative processes. While a few bone-on-a-chip models exist, recapitulating the cellular components of bone, these models do not incorporate the chemical and structural characteristics of bone tissue. Herein, the development of a bone-on-a-chip platform is reported that comprises a 3D structural model of bone. To build the platform, first, a 3D model of bone is produced in a polymer using two-photon polymerization (2PP) from a 3D nano-computed tomography scan of trabecular bone. This 3D model is then coated with a layer of bone mineral-like calcium phosphate. Finally, the 3D bone model is integrated inside a microfluidic device suitable for cell culture. Human menenchymal stromal cells, cultured inside the platform for up to 21 days, show high viability and extensive production of extracellular matrix, rich in collagen. This biomimetic bone-on-a-chip platform can contribute to a better understanding of the processes related to bone formation and remodeling, which in turn can be used for the development of bone regeneration strategies.

1. Introduction

Organ-on-chip (OOC) systems, developed using microengineering and microfluidic technologies, aim at establishing physiological-like microenvironments that recreate the key features of human tissues and organs. Commonly, an OOC device does not reconstitute the full complexity of an organ or tissue, but instead mimics a functional unit, which allows to study certain aspects of its (patho)physiology in a controlled manner. Typically, an OOC device consists of an engineered biomimetic microenvironment, fabricated using biocompatible materials and one or more cell types that are representative of the target tissue, and embedded in a microfluidic setup. Through this convergence of microfabrication technologies and cell biology/tissue engineering, OOC systems offer a promising alternative to the existing preclinical models for drug screening, disease modelling, tissue and organ development, and tissue regeneration.

As such, OOC models are often positioned between the over simplistic conventional cell culture systems that use flat tissue culture plates, and in vivo models, which are associated with ethical implications and financial issues. Moreover, with microfluidic systems, advantageous miniaturization and parallelization can be achieved, which enables an increase in the experimental throughput and work with small amounts of reagents, offering cost-efficiency benefits.

To recapitulate the relevant physiological conditions in vitro, it is important to take into account both the physical and chemical characteristics of the organ/tissue microenvironment. To recreate the physical microenvironment, the properties of the substrate on which cells are cultured as well as the characteristics of the fluid flows (e.g., nutrient supply) to which cells are exposed, can be used as tools. Surface- and bulk properties of the 3D cell culture matrix in an OOC device are important. For example, the parameters such as substrate roughness, curvature, and stiffness have been shown to affect cell behavior. Similarly, the mechanical microenvironment has been shown determinant for mimicking normal physiological functions as well as pathophysiological events in OOC models. Examples include the use of the shear stress, resulting from the medium flow, critical in kidney-on-a-chip models and...
the cyclic deformation of the cell culture substrate to mimic breathing in lung-on-a-chip models.[10,11,20] Concerning the chemical characteristics of the microenvironment, one of the unique and highly valuable capabilities of microfluidics is the controlled handling of fluids that can be used to generate predetermined concentration profiles of soluble species, for example, in the form of gradients. In this context, diffusive mixing from laminar flows without turbulent mixing or thermal convection,[21] as well as assisted mixing using mixers, pumps, and valves,[22] have been extensively explored. Using this unique capability of microfluidics, various models have been developed in which cells were exposed to different concentrations of growth factors[23] or gases, such as oxygen.[24]

From a chemical perspective, bone extracellular matrix (ECM) is a composite material, with collagen type-I as the main Organic component, and calcium phosphate (CaP) in the form of a nanocrystalline non-stoichiometric hydroxyapatite (HA) as the main mineral constituent. HA mineral is found inside collagen fibrils (intrafibrillar mineralization) as well as on their surface.[25,26] Structurally, bone tissue is either compact or trabecular, i.e., porous material, with a porosity of about 80%.[26] Pores with a diameter in the range of 10–100 μm and with struts (pore walls) of about 100 μm in width.[25] Bone ECM is populated by four main cell types being osteoblasts, osteoclasts, osteocytes, and bone lining cells, while the bone marrow niche hosts other cell types such as mesenchymal stromal cells (MSCs), adipocytes, endothelial cells, megakaryocytes, macrophages, and neutrophils.[27]

Unlike many other tissues and organs, few examples exist of on-chip models of bone. Reliable human bone models are, nevertheless, needed in order to obtain a better understanding of bone disease treatments and regenerative therapies. Indeed, while clinical strategies to treat bone-related diseases, such as osteoporosis or bone tumors, and to regenerate large bone defects exist for over 45 years now,[28] the clinical challenges still remain, placing a major burden on the healthcare systems. In 2014, Torisawa et al. developed the first bone marrow-on-a-chip model, where the complexity of bone marrow was recapitulated by first implanting a polydimethylsiloxane (PDMS) construct filled with collagen type-I, demineralized bone powder and bone morphogenetic proteins in mice, and then explanting the device and inserting it into a microfluidic chamber.[29] This novel approach allowed the reconstitution of the physiology of the niche and the complex tissue-level functions of bone marrow, providing a valuable in vitro model for the evaluation of radiation toxicity. Nevertheless, the implantation step in mice contradicts the principle that OOC models should reduce animal testing, and moreover, the model is not applicable to the clinically more relevant human bone marrow. Using a tissue engineering approach, Aleman et al. created a bone marrow-on-a-chip platform containing four cell types present in bone marrow (sinusoidal endothelial cells, arterial endothelial cells, MSCs and osteoblasts) divided into four different compartments and encapsulated in a 3D ECM-derived hydrogel.[30] The authors demonstrated the feasibility of the model to study the interactions of normal and malignant hematopoietic stem and progenitor cells with the cells of the bone marrow model. In a recent article, a chip with two compartments for vascular and hematopoietic cells, respectively, separated by a porous membrane, was developed to predict drug-induced hematopoietic toxicity in human cells.[31] A similar chip design, composed of chambers separated by porous membranes, was used to study bone metastasis induced by breast cancer cells.[32] Like most OOC models, the bone (marrow)-on-a-chip models discussed so far were developed either to study a disease or a therapy. In contrast, bone-related OOC models to study tissue development or regeneration are still scarce. One example is the work by Jusoh et al. who combined HA nanocrystals with fibrin to mimic bone tissue ECM on a chip and used it to study the blood vessel formation by human umbilical vein endothelial cells in this microenvironment.[13]

Here, we report the development of a novel bone-on-a-chip microfluidic platform suitable to study processes related to bone regeneration. We aimed to recapitulate both the physical and chemical characteristics of bone microenvironment. To this end, we used a 3D phase-contrast nano-computed tomography (nanoCT) scan of trabecular bone to fabricate a 3D structural model by employing two-photon polymerization (2PP) laser lithography. 2PP is a direct printing technique that enables the fabrication of structures with sub-micrometer resolution for, i.e., biomedical applications.[34] Previously, we have successfully used 2PP to produce microstructured polylactide substrates to induce morphological changes in osteoblasts outside,[35] and inside a microfluidic chip.[36]

Here, following the fabrication of 3D structural models of bone by 2PP, they were coated with a layer of biomimetic HA to introduce the chemistry of the bone mineral component, and finally integrated into a microfluidic perfusion chamber suitable for long-term cell culture. hMSCs cultured inside the platform for a period of up to 21 days, exhibited limited cell death, and deposited bone-like ECM. This bone-on-a-chip model can be used to study the interactions between cells and bone ECM in a physiological-like microenvironment, which in turn may inform the design of (biomaterials-based) bone regenerative therapies and enable therapeutic studies of bone diseases.

2. Results and Discussion

In the current study, we aimed to develop an OOC model of trabecular bone by converging the techniques from microtechnology, microfluidics, biomaterials science and tissue engineering. While the individual techniques used in this work have been described and applied before, the novelty lies in combining these in such a way that a physiological microenvironment of bone is mimicked. Moreover, taking into account both the chemical and nano- and microstructural properties of the tissue, sets it apart from the more macroscale fabrication techniques and bioreactors frequently used in in vitro bone models.[37] For this, several challenges had to be addressed: 1) the bone nCT image segmentation and optimization for 2PP-printing; 2) deposition of a thin biomimetic CaP coating on a polymer that is not optimized for such a procedure, in order to introduce a bone mineral chemistry into the model, while ensuring that the microstructural features are preserved; 3) designing a microfluidic device and integrating the bone models into it while ensuring that the flow velocity field is minimally affected by the presence of the bone models; 4) long-term culture of clinically relevant hMSCs inside the device; and 5) optimization of high-resolution imaging to allow visualization
of cells, ECM formation and mineralization on a model that is not flat and fully transparent.

2.1. Development of a 3D Model that Recapitulates the Structural Properties of Human Trabecular Bone and the Chemistry of Bone Mineral

To recreate the structural properties of bone tissue, we used a nanoCT image set of human trabecular bone as the design input for high-resolution printing using 2PP. 3D optical stereomicroscopy and confocal laser profilometry results (Figure 1a) indicated that the 3D model reproduced in an acrylic photopolymer exhibited features characteristic of trabecular bone, including trabeculae-like structure with interconnected pores. The printing method proved to be highly reproducible (Figure S1, Supporting Information). In an earlier study, Marino and co-workers used a microCT scan of bone to reproduce the trabecular structure by 2PP, showing that SaOS-2 cells cultured on the structures modified their behavior toward osteogenic differentiation and produced bone-like mineral nodules.\[38\] We used a similar approach here to print 3D bone models obtained from processing nanoCT scans of trabecular bone. Despite the high maximum resolution of the printing technique, the use of a commercial resin and the fact that relatively large bone models were printed (in the range of hundreds of micrometers), limited the resolution of line printing. Other studies also reported resolution to be limited by high printing speed, which is usually required for printing large constructs.\[39\]

Direct printing using 2PP requires the use of a photopolymer that is chemically different from bone tissue. With the aim of introducing a chemistry into the model that is closer to that of bone, we optimized a biomimetic coating method to deposit a thin layer of bone mineral-like CaP on the surface of the 3D polymeric model. No apparent changes of the surface structural properties occurred because of the CaP coating deposition, as was shown by the 3D optical microscopy results (Figure 1a). This was confirmed by the quantification of the laser profilometry results, which showed that values for the surface roughness,

![Figure 1](https://www.advancedsciencenews.com/wi...)

**Figure 1.** Structural characterization of uncoated and calcium phosphate-coated 3D structural models of trabecular bone in a polymer produced using two-photon polymerization laser lithography. a) Stereomicroscope images show a similar structure, although they appear optically different as a result of the presence of the CaP layer, which is opaque. The confocal laser profilometry images appear to be similar for the coated and uncoated model, which is also confirmed by the 3D height maps. b) Quantification of the root mean square height (Sq), reduced valley depth and core material volume, measured over the area occupied by the 3D models show minimal differences between the uncoated and calcium phosphate-coated 3D model. Scale bars = 100 μm.
the depth of the valleys (surface pores), and the core material volume were comparable between the uncoated and coated 3D model of bone (Figure 1b).

The morphological and chemical characteristics of the surface of the coated 3D model was analyzed using an energy-dispersive spectroscopy (EDS) detector coupled to an SEM. EDS spectrum and elemental mapping showed a homogenous distribution of calcium and phosphorus (Figure 2a), demonstrating that the coating was homogenously distributed over the 3D surface. In addition to these elements present in the coating, the spectrum showed the presence of carbon and oxygen, corresponding to the polymer underneath the thin CaP coating. The X-ray diffraction (XRD) pattern of the coating (Figure 2b) was characteristic of a semicrystalline hydroxyapatite (HA). The Fourier transform infrared (FTIR) spectrum (Figure 2c) showed characteristic phosphate bands, as well as the presence of pronounced carbonate bands. This suggests that the apatitic phase was carbonated. The presence of the hydroxyl group belonging to water is plausibly a result of the coating protocol and a relatively short drying time. Previous studies where a similar enzymatic coating method was used reported the production of either HA,\(^{40}\) or a combination of HA and octacalcium phosphate.\(^{41}\) To assess the stability of the coating within the experimental setup, cell culture medium was perfused through the bone-on-a-chip platform (the microfluidic device is discussed in the next section) containing the 3D bone model (without and with CaP coating) over a period of 7 days, collected and analyzed for calcium and phosphorus content using inductively coupled plasma mass spectrometry (ICP-MS) (Figure 2d). While the concentration of phosphorus was similar in both conditions, a significantly lower calcium concentration was observed in the coated condition, showing that the coating was stable over a period of at least 7 days. The observed decrease in the concentration of calcium ions is attributed to the uptake of the ions from the cell culture medium onto the CaP surface, as observed previously.\(^{42}\) Accordingly, a decrease in phosphorus concentration was expected too, because the observed uptake is normally due to precipitation of a new layer of CaP on the surface. Considering that the coating comprised carbonated apatite and that the depletion of calcium, but not of phosphorus, was observed, may suggest the deposition of some calcium carbonate. It should be noted that the on-chip platform used here, in particular its surface-to-volume ratio, is different from the regular cell culture setups used in previous studies, which may be a reason for the different surface dynamics leading to changes in the ionic content of the medium.

In sum, the analysis of the structural and surface chemical properties showed that the 3D model of bone in polymer possesses the trabecular microstructure present in the 3D bone scan, and that the coating method successfully introduced a bone mineral-like CaP layer on the surface, without significantly modifying the surface structure. From a materials science perspective, bone is a composite material, comprising intrafibrillarly mineralized collagen matrix.\(^{39}\) While the deposition of such a matrix, that would make the chemical properties of the model even more closely resembling the natural tissue, may be possible, it would be more complex than the deposition of a mineral layer. Since the mineral component of bone is known to play a major role in bone homeostasis and regeneration, for establishing the
model, we have decided to focus on this component of the bone ECM.

### 2.2. Design, Simulation, Prototyping, and Assembly of the Microfluidic Device Containing the 3D Model of Bone

To enable cell culture on the 3D model of bone under different controlled conditions, a microfluidic device was developed into which the 3D bone model was integrated. The design of the microfluidic device (Figure 3a) included a main chamber with the size of $6.2 \times 3.2 \text{ mm}^2$, that enables integration of one or more 3D bone models having the base area of $800 \times 800 \mu\text{m}^2$. The main chamber was connected to one inlet and one outlet for the perfusion of cell culture medium. Two lateral side channels, separated from the main chamber by an array of pillars, were included as additional access ports for introducing CaP solution and for cell seeding. Moreover, the side channels could be used for additionally supplying soluble factors, such as drugs, to the main chamber, as was shown in an earlier study.[43] Here, these side channels were blocked after depositing the CaP coating and cell seeding, thus creating a dead volume side reservoir, as seen

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**Figure 3.** Design, flow dynamics simulations, and prototyping of the microfluidic device. a) The design of the device comprises a chamber that can host multiple 3D bone models, an inlet/outlet for medium perfusion and two lateral inlets that can serve as additional access ports. b) Simulation of the stationary flow in the empty microfluidic device, performed in COMSOL Multiphysics. When the lateral inlets are blocked, the velocity field shows that the medium flows mainly in the central chamber, while the channels separated by the pillars act as dead-volume side reservoirs. The color map shows the magnitude of the flow velocity and the white arrows the velocity field. c) Simulation of the stationary flow in a microfluidic device containing two bone models. d) Cross-section of the device showing the flow velocity magnitude. e) 3D height map of the PDMS device, produced using soft lithography on the silicon molds. f) Optical microscopy image of the device hosting two 3D bone models.
in the 3D computational flow dynamics simulation (Figure 3b). The incorporation of the 3D bone model did not have an obvious effect on the flow velocity profile in the microfluidics chamber (Figure 3c). The flow was observed in the pores of the 3D model, though at a lower magnitude than in the surrounding area (Figure 3d). Simulations of microfluidic devices containing up to six 3D bone models showed that the flow conditions inside one 3D model are not affected by the presence of other 3D models. This suggests that the bone-on-a-chip platform developed here can be used for performing multiple experiments in parallel (Figure S2, Supporting Information). Upon developing the design and performing simulations, the design was transferred to a film photolithography mask and used for the production of SU-8/silicon molds, with a height of 445 μm (Figure 3e). PDMS replicas were created from the mold and then bonded to a glass slide containing 3D bone models by plasma functionalization, obtaining a gas permeable PDMS platform suitable for cell culture (Figure 3f).

2.3. Human Mesenchymal Stromal Cells Culture in the Bone-on-a-Chip Platform

To validate the suitability of the platform to study the behaviour of cells relevant to bone formation and remodeling in a controlled microenvironment, clinically relevant hMSCs were cultured in the platform for up to 21 days.

First, to assess the attachment and growth, the cells were seeded in the platform, allowed to attach for 24 h without flow and then cultured under continuous medium perfusion at a low flow rate of 100 nl min\(^{-1}\). Immunofluorescence images of Sytox Green (nuclei)/phalloidin (actin)-stained cells after 7 days of culture showed that cells were present on both the 3D bone model without and with CaP coating, homogenously distributed over the surface (Figure S3, Supporting Information).

In another set of experiments, cell viability was assessed after, respectively, 7 and 21 days of culture in the platform. Figure 4 shows cell nuclei (in green) and dead cells (in magenta) superposed on a brightfield image of a 3D bone model. The quantification indicated that at day 7, the viability of cells cultured on the bone-on-a-chip platform was about 70%, independent of the presence of CaP coating on the 3D bone model. A similar percentage of viable cells was observed when cells were cultured in a microfluidic device not containing a 3D bone model (i.e., plain indium-tin oxide [ITO]-coated glass slide), whereas the cells cultured in a device placed over a CaP-coated glass slide showed a viability close to 90%. At 21 days, the percentage of viable cells was close to 90% for both platform with 3D bone model with and without the coating, as well as for the control, i.e., an empty device. Surprisingly, the viability of cells at day 21 in the device built on a CaP-coated cover slide was significantly lower, about 60%. While further investigation is needed to explain this observation, it is possible that the CaP coating thickness on the polymeric 3D bone model and glass slide differed, resulting in differences in concentration of calcium and/or phosphate ions in the medium, which in turn may have affected the cell viability. From these results, it can be concluded that cell culture can be performed using this bone-on-a-chip platform over a relatively long period of time without significant cell death.

Figure 4. Cell viability assessment on bone-on-a-chip platform. a) Live/dead assay (confocal z-projection image), showing nuclei in green and dead cells in magenta, superposed to the bright field image of the 3D bone model. Scale bar: 200 μm. b) Quantification of the cell viability percentage on days 7 and 21, showing the mean and standard deviation of measurements on four samples. Quantification was based on the 3D images rather than on the projection to correct for the overlapping position of cells in the different layers.
Comparatively, in the bone marrow-on-a-chip model developed by Torisawa et al.,[29] cell viability was about 60% after 7 days of culture, although it should be noted that the biological complexity of that system was higher, i.e., different cell types were included in the model. In another bone marrow-on-a-chip system using MSCs and osteoblasts, the cell viability after 7 days of culture was about 60%, but decreased to about 20% at day 10.[30] In the bone-on-a-chip model by Hao et al.,[32] after 30 days of culture, cell apoptosis was observed in around 15% of cells when polycarbonate-PDMS chips were used, and was below 5% in nitrocellulose-PDMS chips. The results of our study showed cell viability that was in the range (or above) the viability reported for other bone/bone marrow-on-a-chip models, confirming the applicability of our device for long-term cell culture studies.

To assess the ability of the cells cultured in the bone-on-a-chip platform to produce ECM, after 7 and 21 days of culture, the devices were opened and the 3D model of bone with and without CaP coating was analyzed using SEM. On day 7, the SEM images (Figure 5, left column) showed that the hMSCs started to colonize the 3D bone-like structure. The cells exhibited a spread morphology and were not only observed on the surface but also inside the pores. No obvious differences were observed between the model with and without CaP coating. For the cell culture after 21 days (Figure 5, right column), SEM images show that the 3D bone-like structure was completely covered with a dense layer of fibrous ECM in both conditions with and without CaP. The ECM layer seemed to expand beyond the 3D bone model. These results, along with the data of live/dead assay demonstrated that hMSCs can be cultured in the device over a period of at least three weeks without significant cell death, and that the cells were able to proliferate and secrete ECM, which are relevant processes leading to new bone formation. Previous studies have shown that the ECM production by hMSCs was more pronounced when cells were cultured in a 3D environment, as compared to 2D culture systems, which may be a reason for the extensive ECM production observed here.[44]

The SEM images further revealed the presence of spherical structures with a diameter of about 5-10 μm in the vicinity of cells cultured on the CaP-coated bone model inside the bone-on-a-chip platform. They were microporous, and, as shown by EDS mapping, contained carbon, calcium and phosphorus (Figure S4, Supporting Information). These structures were similar in size and morphology to mineralized nodules,[45] and resembled those reported previously in another bone-on-a-chip device,[32] and in the Osteoprint model.[38] Based on this result, it is suggested that the cells cultured inside the bone-on-a-chip platform containing CaP-coated 3D bone model were not only able to secrete ECM, but also to mineralize it. This is an interesting observation, since the cells were cultured in basic cell culture medium, thus without stimulators of mineralization such as...
beta-glycerol phosphate, and is plausibly a result of the presence of CaP, which is known to induce the differentiation of MSCs and enhance the mineralization process.\textsuperscript{[46]} Since we observed a depletion of calcium (but not phosphate) from the medium, we cannot exclude the fact that these spherical structures comprised calcium carbonate, and that the phosphorus signal identified using EDS originated from the surrounding area.

To further identify the composition of the formed ECM, 3D bone models with hMSCs cultured on them inside the bone-on-a-chip platform for 21 days were fixed and stained for collagen type-I, which is, together with collagen type-III and collagen type-V, the most abundant constituent of the organic ECM in bone. Indeed, collagen type-I accounts for 90\% of the total collagen in bone.\textsuperscript{[47]} The fluorescence microscopy images (Figure 6) revealed the presence of collagen type-I on both coated and uncoated bone model. The images confirmed that the ECM containing collagen extended beyond the borders of the 3D bone model. Despite THE strong autofluorescence of the polymer used to 3D print the bone model, the visualization of total collagen presence in reflection mode was confirmed.

Collectively, the results of this study showed that by using a combination of high-resolution 2PP lithography, microfluidic and biomaterials/tissue engineering approaches, we have successfully developed a novel biomimetic bone-on-a-chip platform that more closely recapitulates the properties of bone than the existing in vitro bone models. 2PP was successfully used to build a 3D model resembling the microscale features of trabecular bone. The bone mineral-like chemistry was introduced on the surface of the 3D model using a biomimetic precipitation technique and finally, the 3D bone model was integrated inside a microfluidic device through which cell culture medium is perfused. Therefore, our model combines various biomimetic elements of the bone microenvironment, i.e., structure (3D model), chemistry (CaP coating), and physiology (medium flow).

It is, nevertheless, important to note that during processing and printing of the 3D model using 2PP, a 4.16× scaling effect of the nanoCT scan was observed, derived from the adjustment of the scan voxel size to \( \approx 1\ \mu m^3 \) by the DScribe software conversion, which automatically optimizes the models for printing with commercial resins. Because of this mismatch between the very high-resolution bone morphology data obtained using nanoCT and the capabilities of the 2PP lithography with the selected resin, the microstructural properties of bone were reproduced, however, with the aforementioned scaling factor. Considering the fractal nature of bone ECM,\textsuperscript{[48]} which begins at the nanoscale and extends to the microscale,\textsuperscript{[49]} further optimization of the technique is required to cover this entire scale range.\textsuperscript{[25]} A step toward the creation of a model that more accurately resembles the original nano/submicron features of bone lies in the development of optimized resins that allow printing with voxel size comparable with the scanning pixel dimensions. To mimic the chemical microenvironment, these efforts should focus on the use of photocrosslinkable collagen mixtures\textsuperscript{[50]} and subsequent collagen biomineralization strategies.\textsuperscript{[26]} Recently, potentially interesting methacrylate collagen resins for the use in stereolithography and digital light processing have been reported, such as CoLMa from Cellink,\textsuperscript{[51]} and Photocol from Advanced Biomatrix.\textsuperscript{[52]} While this is an obvious advancement.

![Figure 6. Production of collagen on the bone-on-a-chip platform at day 21. Staining performed for collagen type-I (in red) and total collagen (in yellow, obtained using microscope reflection mode at 488 nm) on uncoated and calcium phosphate-coated 3D bone model. Overlay is shown for total collagen outline overlapping collagen type-I signal. Scalebars = 200 μm.](image-url)
in the development of a next-generation platform, the synthesis of a collagen-based ink that is both suitable for printing using nanolithography techniques, taking advantage of their high resolution, and amenable for intrafibrillar mineralization is not a trivial challenge.\cite{53}

Another important aspect of the bone microenvironment that is worth exploring concerns mechanical properties. For example, by more closely recapitulating the chemical composition of bone in the model, the mechanical properties of the substrate, such as stiffness, will also be adjusted. The substrate stiffness has been shown to affect various aspects of cell-material interactions including cell attachment, growth, and differentiation\cite{17,54} therefore, it is important for mimicking the process of bone formation and remodeling. In the current model, the underlying polymer had a stiffness of 2.1 \pm 0.3 GPa\cite{55} and was coated with a thin ceramic layer that is stiff and brittle. The elastic modulus of HA has been reported to be in the range of 120–130 GPa\cite{56}, whereas the stiffness of biomineralized collagen is around 0.177 \pm 0.031 GPa\cite{57} thus an order of magnitude lower. Comparatively, the stiffness of natural trabecular bone lies between that of the polymer used here and the mineral component alone, and has been reported to be between 5 and 19 GPa\cite{58} in wet conditions, with different values for different bones.\cite{59} Also in the context of mechanical properties, development of new resins, with stiffness values closer to those of bone ECM, will be relevant.

Apart from the mechanical properties of the material itself, the cyclic mechanical loading most bones are subjected to is an important aspect worth studying, since the cyclic loading has been shown to have an effect on bone tissue development.\cite{59} For example, the use of an alternative resin allowing for elastic deformation of the 3D bone models could be advantageous, as well as the introduction of a mechanical actuator, similar to what was used in lung-on-a-chip devices.\cite{3,10}

We have demonstrated that the bone-on-a-chip platform developed here enabled a long-term culture of hMSCs. By integrating the 3D bone model inside the microfluidic device, it was possible to continuously supply the cells with a flow of cell culture medium, overcoming the limitations of conventional cell culture systems where the concentrations of nutrients and metabolic waste secreted by the cells oscillate between the manual medium refreshment time points. While in this study, we used a low flow rate, exerting a low shear stress on cells, the setup allows the introduction of different fluid shear stresses. This is relevant in the context of models for bone regeneration, as shear stress has been proposed as one of the main mediators of cell activity and bone tissue formation.\cite{60}

This bone-on-a-chip platform also offers the possibility for additionally introducing different compounds in the solution, such as growth factors or small molecules, to the cells cultured on the 3D bone model. Moreover, this platform offers the possibility for studying the effects of chemical and physical cues on the cell behavior in a combinatorial manner, which is still difficult to achieve in many existing in vitro models.\cite{44} Taken together, the different flow regimes that can be created inside the platform owing to its microfluidic nature, are to be exploited to their maximum extent in future studies.

The cell culture experiments further showed that the platform with the integrated 3D bone model enabled the cells to proliferate and eventually produce abundant ECM comprising collagen. ECM provides cells with structural and mechanical support\cite{61} and influences their behavior, including the differentiation of hMSCs toward the osteogenic lineage.\cite{62} Therefore, this platform can be used to study the ECM development under near-physiological conditions, and to test different biomolecular interactions. The observation that the ECM formed beyond the borders of the 3D bone model (Figure S5, Supporting Information) is interesting, as it suggests that the platform could be used for studying a bone defect closure, e.g., by introducing an artificial defect inside the 3D bone model. Similarly, several modifications to the 3D model can be introduced to increase its porosity (e.g., to mimic the thinning of trabeculae in the case of osteoporosis) or resemble microfractures occurring after a trauma. On the other hand, the use of different (patient-derived) cell types, including potential cocultures, can be used to study processes relevant to bone repair/regeneration or to diseases like unbalanced remodeling in the case of osteoporosis.

While in this study we focused on the evaluation of the cell viability and ECM production for providing a proof-of-concept, other aspects of the bone formation process such as cell attachment and shape and the production of proteins relevant to bone regeneration, e.g., bone morphogenetic protein 2 or osteopontin, can be quantified by immunohistochemistry and confocal microscopy. By fabricating devices that contain multiple 3D bone models, and thus allow for more cells to be cultured, the more conventional assays to study cell fate, such as polymerase-chain reaction analysis (PCR) or enzyme-linked immunosorbent assay (ELISA), can be performed.

Finally, owing to its microscale, our platform is also suitable for live microscopy, using, e.g., spinning-disk or confocal microscopes. This makes it interesting for real-time dynamic studies cell response inside a 3D bone-like environment to, treatments such as drugs-carrying nanomaterials,\cite{63} extracellular vesicles,\cite{64} or microRNA-based gene therapy.\cite{65}

3. Conclusions

In conclusion, we fabricated a microscale 3D model containing the main structural features of trabecular bone (i.e., surface morphology and porosity) in a polymer using 2PP, and modified its surface chemistry with a semicrystalline CaP layer to resemble that of bone mineral. The integration of this bone model inside a microfluidic device enabled long-term culture of hMSCs, sustaining high cell viability and production of abundant ECM, containing collagen type-I. This novel bone-on-a-chip platform is a potentially useful tool for studying processes relevant to bone regeneration in a physiological-like microenvironment.

4. Experimental Section

**Optimization and Printing of 3D Model of Bone:** The 3D model reconstruction started from a set of phase-contrast nanoCT scans of natural trabecular bone acquired with a 240 nm resolution.\cite{66} The nanoCT images (Figure 7a) were loaded into the CtAn software (Bruker), from which a volume was extracted, thresholded and converted into an STL file (Figure 7b). The STL was processed in Meshlab\cite{67} in order to reduce the file size and eliminate unnecessary parts of the scan, such as isolated bone microfragments or artifacts (Figure 7c). The reduction was achieved.
Figure 7. Schematic representation of the acquisition and processing workflow from nano-computed tomography images into a volumetric model converted for 3D printing. A stack of images of a trabecular bone is acquired by high-resolution nano-computed tomography and b) converted into binary images, removing unspecific signal by fine thresholding. c) Then, a 3D model is reconstituted and optimized for printing. d) Finally, a clean and conservative 3D-model is available for printing with direct laser writing by two-photon polymerization laser lithography.

by using the quadric edge collapse decimation algorithm\(^{[68]}\) with priority both on preserving the normal angle of the mesh faces and the topological organization of the mesh elements, and on cleaning by removing the isolated pieces with diameter below 1 \(\mu m\). The STL model was further processed in DScribe (Photonic Professional) to optimize it for printing using 2PP lithography (NanoScribe GT, Photonic Professional). In this step, a 4.16x scaling factor was introduced, due to the software adjustment of the scan voxel size to 1 \(\mu m^3\) for optimizing the printing results with commercial resins.

The bone model had a base area of \(800 \times 800 \mu m^2\), and a height of 100 \(\mu m\). The printing parameters were defined to have an interlayer distance of 600 nm and a hatching distance of 500 nm. Writing was performed in the galvo scan mode and the 3D model was divided in sub-blocks, the size of which corresponded to the maximum allowed size in one print step. The 3D model was divided into sub-blocks and resubmitted for optimization, increasing the efficiency of the process. In total, the printing process was finalized, the slides were developed in propylene glycol monomethyl ether acetate (PGMEA, ReagentPlus \(\geq 99.5\%), Sigma-Aldrich\), followed by rinsing with ultrapure isopropanol and gentle blow-drying.

**Calcium Phosphate Layer Deposition:** For depositing a thin layer of CaP onto the 3D bone model, a slightly modified method published earlier was used\(^{[40,41,70]}\). This method is based on the enzymatic degradation of urea by urease, resulting in a slow increase of the pH of a solution containing calcium and inorganic phosphate ions, and in turn the precipitation of CaP by urease, resulting in a slow increase of the pH of a solution containing calcium and inorganic phosphate ions, and in turn the precipitation of CaP. The calcium-to-phosphorus ratio was adjusted to 1.5, the urea concentration to 200 mM and the units of urease to 1 U ml\(^{-1}\). The 3D model of bone was placed on the bottom of a glass beaker prior to the addition of urease to the CaP solution and left overnight. The coated model was then thoroughly washed with MilliQ water.

**Characterization of the 3D Bone Model:** The structure of the 3D bone model before and after CaP coating was characterized by optical brightfield microscopy and laser profilometry. A z-stack of images was acquired using a Nikon SMZ25 stereomicroscope with a step size of 2 \(\mu m\), and reconstructed into a 3D image using the Nikon’s NIS Elements software. The samples were also characterized with a confocal laser scanning optical profilometer (Keyence VHX-200, Keyence, Japan) using a 20x objective and XY stacks. The profilometry data were processed using the Keyence Multifile Analyzer software, setting the zero level on the surface of the glass slides, and measuring the root mean square height, reduced valley depth and core material volume on the area occupied by the 3D bone model. These parameters are recommended in ISO 25178 standard for roughness measurements. The root mean square height expresses the root mean of the square of the difference of height of each point on the surface compared to the arithmetic mean height of the surface, and it was used to evaluate surface roughness. The reduced valley depth is a measure of the valley depth below the core roughness, and it is also an indicator of the depth of the area in which a fluid applied to the surface may accumulate. The core material volume is the volume of the material comprised between 10% below the highest peak and 80% above the deepest valley, and it informs about the volume of the material excluding the highest peaks and deepest valleys; it also represents the part of the surface material that would not interact with another surface when in contact.\(^{[71]}\)

Concerning the chemical characterization, the elemental composition of the coating deposited on the 3D printed bone model was characterized using an EDAX detector (EDAX) coupled to a SEM (Philips, XL30). The images were acquired at 10 mm distance from the sample and a voltage of 15 kV. Elemental maps and spectra were copied from the EDAX software report.

The powder scraped off the surface after deposition on ITO-coated glass slides, similar to the ones used for printing the 3D models, was collected, washed, dried, and analyzed by XRD (Bruker D2 Phaser) with a step size of \(2\theta\) = 0.02°, one second per step and one rotation per second, in the interval \(2\theta = 5°−70°\). The data was analyzed using Match! V.3 and correlated with the COD database for pattern matching. The powder was further characterized by FTIR (Nicolet i50, Thermo Fisher) in attenuated total reflection mode, with 2 nm resolution, averaged over 32 measurements. The stability of the coating was assessed by collecting the cell culture medium that was flown for 7 days through the bone-on-a-chip platform, containing either uncoated or CaP-coated 3D bone model, and measuring the concentration of calcium and phosphorus by ICP-MS (iCap Q, Thermo Scientific).

**Microfluidic Chip Design:** The microfluidic chip was designed to host up to six 3D bone models. The design included one inlet for cell culture medium perfusion and one outlet, as well as two lateral inlets, that may in the future be used to add additional soluble species to the chamber with 3D bone models.

The 2D design was made in Clewin 5 (WieWeb, Netherlands), exported to a DXF file and imported into COMSOL Multiphysics 5.4. The 2D design was converted into a 3D representation of the chamber using the extrusion tool. The 3D model was used to simulate the velocity field in the chamber when the flow in the inlet was 100 nL min\(^{-1}\), assuming laminar stationary flow.

The design was printed in a high-resolution film mask (Micro Lithography Services, UK) and used to produce silicon masters of the device. Silicon wafers (type P doped with boron, Si-Mat Silicon Materials, Germany) with a diameter of 100 mm and a thickness of 525 ± 25 \(\mu m\), were used as substrate to deposit a uniform layer of SU-8 100 (Micro Resist Technology, Germany) by spin-coating, followed by pre-baking, exposure to UV light and post-baking. The wafers were developed in PGMEA (ReagentPlus \(\geq 99.5\%), Sigma-Aldrich\), followed...
by rinsing with ultrapure isopropanol and blow-drying. After the development, hard baking was performed to harden the structures and avoid the formation of microcracks. The structural properties of the wafer were characterized by confocal laser profilometry, in top-transparent film mode.

To fabricate the devices, PDMS (Sylgard 184) was mixed in 1:10 ratio with crosslinker, cast on the wafers, degassed under vacuum and baked at 80 °C for 2 h. The structural properties of the PDMS chamber were characterized by confocal laser profilometry, as described for the 3D bone model above. The devices were then bonded to glass slides using plasma bonding, at the power of 50 W during 4 min. Prior to the plasma-bonding process, the outer part of the coated slides was cleaned with 1% HNO₃ solution to facilitate the bonding. The devices were then baked in an oven at 78 °C for 2 h.

Cell Culture and Characterization of Viability and Extracellular Matrix Formation: hMSCs were obtained from human bone marrow aspirates of one healthy donor, after written informed consent, and expanded in basic cell culture medium containing alpha-MEM without nucleosides, 0.2 mM ascorbic acid 2-phosphate and 10% fetal bovine serum, in huminified atmosphere in an incubator at 37 °C with 5% CO₂. The cell culture medium was refreshed every 2 days. At passage 2, the cells were trypsinized and stored in liquid nitrogen until further use. After thawing, the cells were expanded again in basic cell culture medium and trypsinized at 70% confluence for the seeding on the chips. The seeding was done by slowly introducing 50 of medium containing 500,000 cells mL⁻¹ (25 cells per chip) with a micropipette. The inlets were blocked with pipette tips to avoid medium evaporation, and allowed to attach for 24 h in a humidified atmosphere in an incubator at 37 °C with 5% CO₂. Then, the devices with cells were moved to another incubator and connected to a microfluidic pump via tygon tubing (Ultratune, Harvard Apparatus) that supplied the devices with continuous flow of basic cell culture medium supplemented with 10 U mL⁻¹ penicillin/streptomycin at 100 mL min⁻¹.

After 7 and 21 days of culture, the PDMS chamber was carefully removed and the 3D bone models with cells were transferred to a well plate, where they were stained for 30 min with live/dead Fixable Far Red Dead Cell Stain (Thermo Fisher Scientific) at the concentration of 1 μL mL⁻¹ as recommended by the manufacturer, at room temperature. They were then washed with PBS, fixed for 24 h with 4% PFA, washed once with PBS with 1% bovine serum albumin (BSA), then with PBST (PBS + Tween 20, 0.1% v/v) to reduce dye adhesion to the substrate, and finally washed again and stored in PBS.

To the strong autofluorescence at short wavelengths of the material used for building the 3D bone model using 2PP, the use of the commonly applied DAPI staining was not possible. Therefore, the nuclei of the cells were stained using Sytox Green Nuclei Acid Stain (Thermo Fisher Scientific) diluted 1:7500 in Tris buffer saline for 2 h, followed by subsequent washing steps with PBST and PBS, and storage of the samples in PBS.

To analyze the expression of collagen type-I, the samples were permeabilized in 0.1% TritonX for 10 min, blocked for 1 h in 1% BSA in PBS and then stained with anti-collagen type-I antibody (Abcam ab34710). The primary antibody was left to incubate overnight at 4 °C, followed by three wash steps in PBS, incubation with secondary antibody (1:250 goat anti-rabbit Alexa 568 in 1% BSA in PBS) for 2 h at room temperature, washing once in PBST and twice in PBS, before storing in PBS for later imaging. The samples were transferred to a microslide well (Ibidi), covered by PBS, and imaged using a confocal microscope (Leica TCS SP8 STED). The samples were imaged using time-gating with hybrid detectors to minimize the signal coming from the substrate. The samples were also imaged in reflection mode at 488 nm to evaluate the presence of collagen.¹³

After the immunofluorescence imaging, the samples were first dehydrated in ethanol series (30–100% EtOH), then treated with hexamethyldisilazane (HDMS) overnight and finally coated with a 3 nm iridium layer using a sputter coater (Quorum Q150T ES Plus). The samples were imaged in a high-resolution SEM (Teneo, FEI) at 2 kV voltage and 10 mm sample distance. The areas of the samples that contained mineralized nodules were imaged in an environmental SEM equipped with EDS detector (Versa 3D Dual Beam, FEI) at 15 kV and 10 mm sample distance.

Data Processing and Statistical Analysis: The results were processed using Graphpad Prism v.9.3.0. Comparisons between the sample groups were done using the one-way ANOVA with the cases of the laser profilometry and ICP-MS data, and two-way ANOVA for the live/dead quantification. In both cases, Tukey’s post-hoc test was used, and the significance level was set at p < 0.05.

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.

Author Contributions
V. P.-G.-C. conceptualized the article, designed and performed the experiments, analyzed the data and wrote the manuscript; A.Z. participated in performing the experiments and analyzing the data; B.H. and M.B. provided resources (nanoCT data) and revised the article; P.H. conceptualized the article, acquired funding, supervised the project, wrote and revised the manuscript; D.B. conceptualized the article, designed the experiments, provided technical guidance, supervised the project, helped with data visualization, and revised the manuscript.

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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