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Water-in-PDMS Emulsion Templating of Highly Interconnected Porous Architectures for 3D Cell Culture

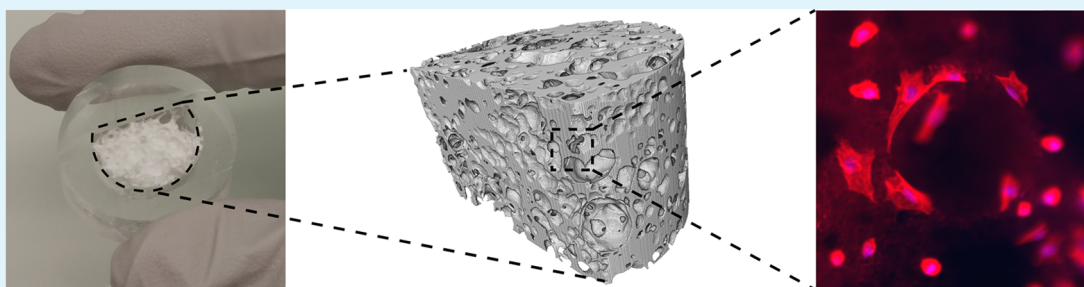
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ABSTRACT: The development of advanced techniques of fabrication of three-dimensional (3D) microenvironments for the study of cell growth and proliferation has become one of the major motivations of material scientists and bioengineers in the past decade. Here, we present a novel residueless 3D structuration technique of poly(dimethylsiloxane) (PDMS) by water-in-PDMS emulsion casting and subsequent curing process in temperature-/pressure-controlled environment. Scanning electron microscopy and X-ray microcomputed tomography allowed us to investigate the impact of those parameters on the microarchitecture of the porous structure. We demonstrated that the optimized emulsion casting process gives rise to large-scale and highly interconnected network with pore size ranging from 500 μm to 1.5 mm that turned out to be nicely adapted to 3D cell culture. Experimental cell culture validations were performed using SaOS-2 (osteosarcoma) cell lines. Epifluorescence and deep penetration imaging techniques as two-photon confocal microscopy unveiled information about cell morphology and confirmed a homogeneous cell proliferation and spatial distribution in the 3D porous structure within an available volume larger than 1 cm^3 . These results open alternative scenarios for the fabrication and integration of porous scaffolds for the development of 3D cell culture platforms.

KEYWORDS: PDMS, 3D scaffold, emulsion, porosity, osteosarcoma cells

1. INTRODUCTION

23 The development of porous materials has been a major
24 concern for materials science since decades. Their properties
25 play an important role in many applications such as energy
26 storage and conversion, pollutant gas capture, and drug
27 delivery.^{1–4} Porous structures are also fundamental in the
28 development of living organisms. Oxygen capture in our bodies
29 is due to the porosity of alveolar tissue in our lungs, which
30 maximizes the exchange surface available for this task.⁵ In
31 bones, the trabecular topology works as a niche for the bone
32 marrow and provides a proper environment for cellular
33 regeneration.⁶ Further, in the context of the realization of
34 biomimetic scaffolds for cell culture and tissue engineering
35 studies, the accurate tuning of pore distribution and pore size
36 allows the cells to infiltrate easily within the material, promote
37 the perfusion of nourishment, and facilitate the vascularization
38 of the restored tissue.⁷ To achieve these topologies, many

solutions have been recently proposed in the field of material
sciences and, in particular, polymer science.

One of the most extended and widely used materials for
bioapplications in the last decades is poly(dimethylsiloxane)
(PDMS).⁸ Since Wacker Chemie discovered this silicone-
based organic elastomer in the 1950 s, it has found a large
range of applications starting from lab on chips and
microfluidic devices⁹ to contact lenses, medical devices,^{10,11}
alimentary industry, passing through energy storage,¹² flexible
electronics,^{13–15} and piezoelectric actuators.¹⁶ PDMS is also
known for its biocompatibility¹⁷ and molding properties¹⁸ to
generate medical devices or even bioimplants.¹⁹ Furthermore,
PDMS features a low surface tension and energy, and it is

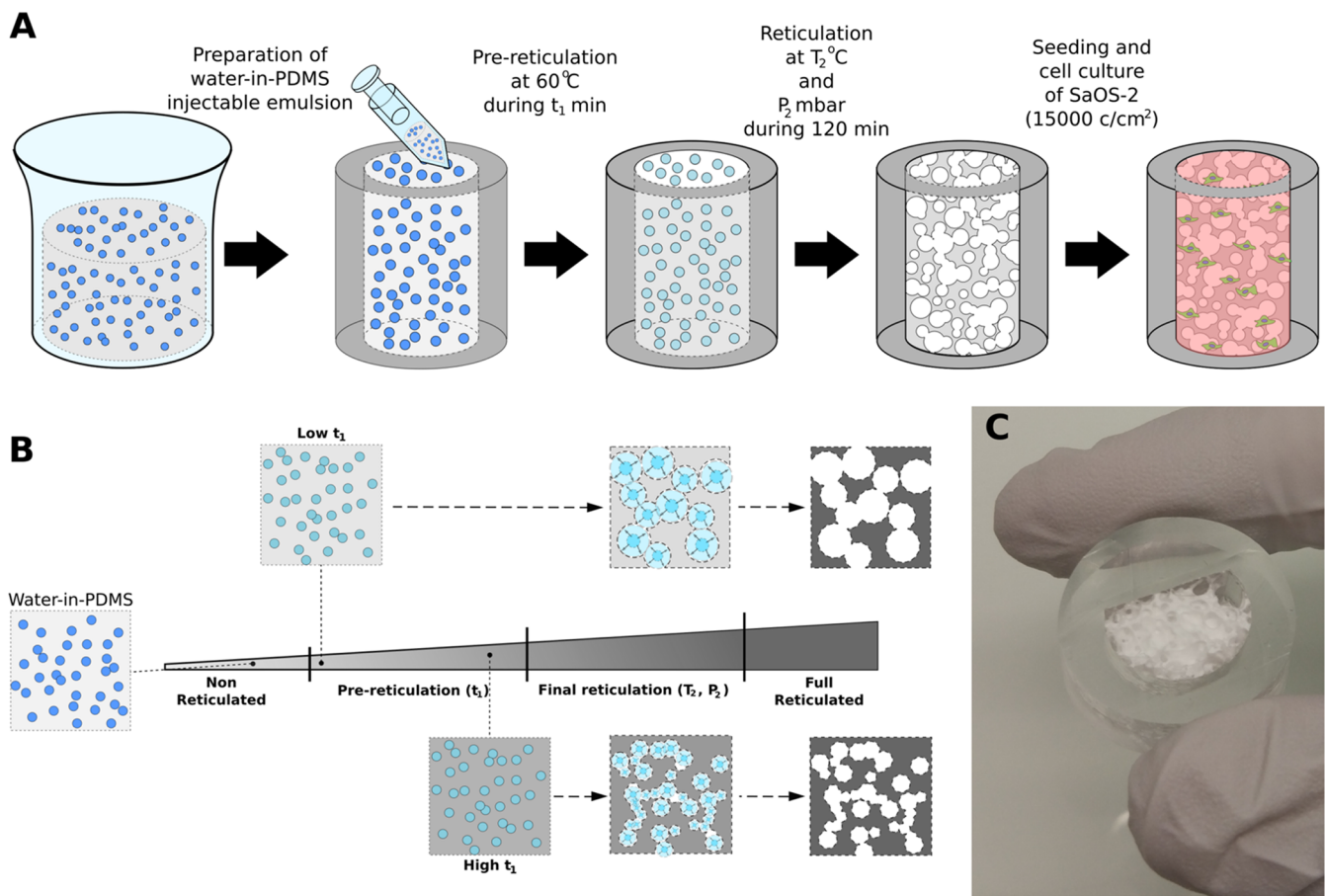


Figure 1. (A) Sketch of the fabrication process of the PDMS porous scaffold. The emulsion is injected into a PDMS shell and placed in an oven at 60 °C for t_1 min. Afterward, the scaffolds are transferred to a vacuum oven at temperature T_2 under pressure P_2 . (B) Sketch of the impact of the physical parameters of the fabrication process in the porous morphology. (C) Optical image of the PDMS porous scaffold.

52 hydrophobic although its surface properties are easily tunable
 53 via oxygen plasma treatment to introduce hydroxyl groups,
 54 allowing grafting of proteins or other functional groups.²⁰
 55 Concerning biological applications, PDMS is well adapted
 56 with cell biology applications:²¹ it is compatible with almost
 57 every technique of protein coating for cell adhesion, and its
 58 mechanical properties^{18,22} are known to be compatible with
 59 cell culture. One of the main advantages over other materials is
 60 its permeability to oxygen and water, which allows the cell
 61 medium to oxygenate and reach biocompatibility levels.²³ In
 62 addition, it is transparent and compatible with optical
 63 characterization method and is lowly photoluminescent,²⁴
 64 allowing the use of fluorescent markers for the visualization of
 65 cellular features. For all of these reasons, PDMS is broadly
 66 used in biological applications and is undoubtedly one of the
 67 main materials used in the fabrication of health sensors, flexible
 68 biocontacts, or microfluidic devices for biomedical applica-
 69 tions.

70 Nowadays, one of the main challenges in tissue engineering
 71 is to develop models of microenvironments that mimic the key
 72 aspects of the architecture and organization of living tissues.
 73 Hence, in the past two decades, we witnessed a transition from
 74 conventional two-dimensional (2D) Petri dish monolayer cell
 75 culture approaches to three-dimensional (3D) architectures
 76 featuring topological, mechanical, and biochemical aspects
 77 matching the natural growth environment of cells.^{25–28} To
 78 fulfill this need, material scientists, biomedical engineers, and
 79 microfabrication researchers started to investigate and develop

protocols aiming at realizing such architectures by exploiting
 diverse additive manufacturing and other 3D fabrication
 techniques such as fused deposition modeling and electro-
 spinning,²⁹ stereolithography,^{30,31} direct laser writing,^{32–35}
 or bioprinting.³⁶ Although most of these approaches allow the
 fabrication of scaffold features down to the micrometric or
 even submicrometric scale, they are often limited by the overall
 printable size of the object, by the cost of the fabrication setup,
 as well as by the scarcity of biocompatible materials for
 biological applications.³⁷ PDMS can be hardly integrated
 within additive manufacturing processes, and it is not possible
 to unmold three-dimensional patterns with cell resolution
 while its biocompatibility for biomedical applications is widely
 proved. On the other hand, a large community of chemists and
 material scientists developed fabrication protocols of PDMS
 sponges based on emulsions/foams,³⁸ gas foaming,³⁹ or
 microcasting of sacrificial materials/structures.^{40–43} The
 important molecular role of the hydrophobic/hydrophilic
 tails and the tuning of its wettability properties⁴⁴ make it a
 perfect candidate for microfluidic or environmental applica-
 tions,⁴⁵ while some groups have recently tested the possibility
 of using PDMS macroporous sponges for tissue engineer-
 ing.^{46,47}

In this paper, we report for the first time a simple, rapid, and
 cost-effective 3D fabrication technique for creating mesoscale
 porous PDMS scaffolds of centimeter scale, featuring a pore
 size ranging from millimeter to micrometer scale. The protocol
 is based on H₂O/PDMS emulsion casting and subsequent

108 pressure-/temperature-controlled curing. This process is
109 similar to previously reported polymer high internal phase
110 emulsion (polyHIPE) technique;^{48,49} however, it relies on a
111 progressive expansion of the internal phase that allowed us to
112 tune the pore size, distribution, and interconnectivity within
113 the architecture. Optical microscopy, scanning electron
114 microscopy (SEM), and X-ray tomography investigations
115 showed an interconnected porous architecture with an
116 available porosity estimated to be higher than 57%. The
117 scaffolds were then tested in the presence of an osteosarcoma
118 cell line, namely, SaOS-2, which holds several osteoblastic
119 features⁵⁰ and is commonly employed as an in vitro model for
120 studying the transition of human osteoblasts to osteocytes.⁵¹
121 The SEM, fluorescence, and two-photon confocal imaging
122 characterizations of both the surface and the inner core of the
123 scaffold revealed an efficient 3D cell colonization of the
124 architecture and the presence of the typical flattened
125 cytoskeletal morphology expected for SaOS-2 cells. The results
126 show how the fast prototyping fabrication protocol that we
127 developed and the tunability of the pore size and pore
128 distribution open a promising scenario for the development of
129 3D cell culture models and tissue engineering applications
130 involving PDMS.

2. MATERIALS AND METHODS

131 **2.1. Materials.** Poly(dimethylsiloxane) (PDMS) was purchased
132 from Dow Corning in a kit containing a silicone base and a curing
133 agent (Sylgard 184). All of the PDMS mixtures presented in this work
134 were prepared following standard proportions (10:1 = base/curing
135 agent w/w) and properly degassed using a dedicated chamber under
136 vacuum. We employed ultrapure (type I) deionized water (DIW)
137 from a Milli-Q Direct purifier system. Molds were fabricated with a
138 3D stereolithographic system DWS 29J+ in DS3000 and DL260
139 materials from DWS Systems.

140 **2.2. Fabrication of Standardized PDMS Scaffold Holders.**
141 Due to the biological purposes of this work, the dimensions of all of
142 the developed scaffolds were designed to be compatible with standard
143 cell culture consumables configuration. The samples were fabricated
144 to fit inside a 12-well plate (22 mm diameter for each well). To ensure
145 a perfect sealing of the scaffold in the wells and provide a manageable
146 object, a PDMS scaffold holder was fabricated by 3D printing and
147 inserted in the multiwell plate during the casting (see Figure S1 in the
148 Supporting Information) to obtain a cylindrical shell of PDMS with a
149 semicircular empty space where we then injected the water/PDMS
150 emulsion. The height of the scaffold holder was set between 1 and 1.5
151 cm, while its inner diameter was set to 1.4 cm. PDMS silicone was
152 poured on the plate and cured at 60 °C overnight.

153 **2.3. Emulsion Casting.** Highly porous PDMS scaffolds were
154 fabricated by the method of emulsion casting, using water as internal
155 phase, and reticulating the emulsion within specific environmental
156 conditions (Figure 1A,B). Water-in-PDMS emulsion was made by
157 progressively adding small quantities (~10% of PDMS mass) of DIW
158 and mixing until reaching 70% of water-in-silicone. With this process,
159 we generated a water-in-silicone emulsion that was then injected into
160 the cylindrical sample holder. The reticulation process consisted of
161 two separate steps of reticulation by varying temperatures and
162 pressures that allowed us to control the pore size and distribution
163 (Figure 1C): (1) the samples were first placed inside an oven
164 (Memmert) at $T_1 = 60$ °C for a specific time t_1 ranging from 30 to 60
165 min in atmospheric pressure condition; (2) the samples were then
166 transferred into a vacuum oven (SalvisLAB Vacucenter) at T_2 ranging
167 between 110 and 130 °C with an absolute pressure value P_2 of 400 or
168 700 mbar for 2 h. After this time, we did not observe any notable
169 evolution of the size and interconnectivity of the pores. Both edges of
170 the samples were removed by slicing the cylinder with a surgical blade
171 to obtain a clean surface.

2.4. Scanning Electron Microscopy. We used scanning electron
172 microscopy (SEM) to observe the microscopic morphology of the
173 samples with a Hitachi S-4800 system at an accelerating voltage
174 ranging from 2 to 5 kV and 10 μ A current. The imaging was
175 performed on core regions of the scaffolds, accessed by cross-cutting
176 the samples with a surgical blade, in flat and 45° tilt-angle SEM
177 sample holder positioning configuration. To improve the resolution
178 and avoid charging effects, the samples were coated with a 15 nm
179 layer of sputtered gold using PECS I from Gatan Systems. False-color
180 imaging treatment was performed by using the open-access software
181 Gimp 2. 182

2.5. X-ray Tomography. To investigate the pore properties of the
183 sample, X-ray microcomputed tomography (μ CT) imaging was
184 performed. μ CT is a nondestructive imaging technique that allows
185 quantification of internal features of an object in three dimensions
186 with microscopic resolution. In this study, the specimen was inserted
187 inside a X-ray microtomography machine manufactured by RX
188 Solutions (EasyTom XL 150). A sealed-type microfocus X-ray source
189 with beryllium target was used. The X-ray source energy was adjusted
190 to the resolution of the scan: the source voltage was fixed at 66 kV
191 and source current at 268 μ A. Before the acquisition, standard black
192 and gain calibrations were performed. A complete scan was acquired
193 by recording 1440 projections of the sample at different angles,
194 equally spaced on 360°, with a flat panel of 1920 \times 1536 pixels. Each
195 projection had average exposure times of 0.11 and 5 s. The 3D
196 volume and corresponding slices were reconstructed with the RX
197 Solutions software, X-Act, using a filtered back-projection algorithm.
198 Reconstructed slices had an isotropic resolution of 18 μ m. 199
200 Postprocessing of images was performed with Avizo 9.7.0, a software
201 dedicated to data visualization, segmentation, and quantification. A
202 nonlocal means filter⁵² was first used to remove noise. For the
203 binarization of images, two different methods were used: (1) a user-
204 defined threshold was applied to separate pores and PDMS matrix
205 and extract the binary image on each slice; (2) a watershed
206 algorithm⁵³ was also applied on filtered images to separate pores
207 and PDMS material. Watershed-based segmentation consists of
208 transforming the gray-level image as a topographic map, where high
209 intensity represents peaks and hills while low intensity represents
210 valleys. The obtained topographic image is then flooded, starting from
211 user-defined seeds, using an automatic gradient magnitude algorithm.
212 Dams are built to avoid merging water from two different catchment
213 basins. The segmentation result is defined by the locations of the
214 dams, i.e., the watershed lines. Porosity in both cases (user-defined
215 threshold and watershed algorithm) was finally calculated as the
216 fraction of pore volume over the total volume of the specimen. The
217 connectivity of pores was evaluated with the Axis Connectivity
218 function available on Avizo.

2.6. Water Retention. To complement the results obtained by X-
219 ray microcomputed tomography, an empirical test was completed to
220 provide experimental data about the absorbance of water within the
221 3D architecture. The porous PDMS scaffolds were dried in a vacuum
222 oven at 60 °C overnight and then weighted to obtain W_{dry} . The
223 PDMS was plasma-activated using oxygen plasma treatment (Diener
224 Electronic, 5 sccm oxygen flow, 0.5 mbar, 5 min, 50 W) to enhance
225 wettability and immediately soaked in PBS over 48 h. The water
226 retained in the scaffold was measured using an electronic balance that
227 provided us with W_{wet} . The percent of water remaining in the porous
228 PDMS scaffold was calculated as⁵⁴ 229

$$\text{water retention (\%)} = \frac{(W_{wet} - W_{dry})}{W_{dry}} \times 100$$

2.7. Cell Culture, Fixation, and Staining. Prior to cell culture, 230
231 the porous PDMS scaffolds were first sterilized for 1 h under UV
232 exposure at 254 nm. The PDMS surface was then activated with an
233 oxygen plasma treatment (Diener Electronic, 5 sccm oxygen flow, 0.5
234 mbar, 5 min, 50 W) and coated with 10 μ g/mL of human fibronectin
235 (Corning) for 1 h at room temperature. The osteosarcoma cell line
236 (SaOS-2) was obtained from the American Type Culture Collection
237 (ATCC) and grown using minimum essential medium α (α -MEM) 237

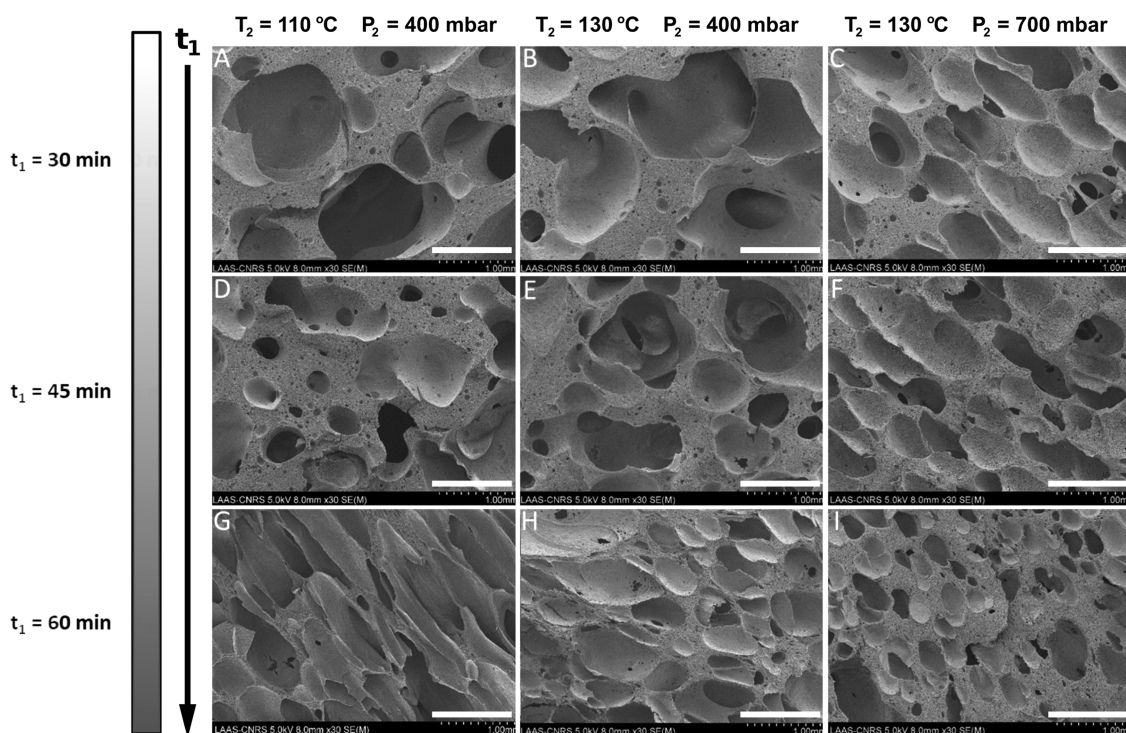


Figure 2. SEM characterization of the cross-sectional regions in the PDMS porous scaffold for different curing parameters. Along the vertical axis, we vary the time t_1 for the prereticulation stage at 60 °C. Along the horizontal axis, we vary the temperature T_2 and the pressure P_2 of the second reticulation process. The scale bar is 1 mm.

238 containing nucleosides, GlutaMAX, (Gibco, Fisher Scientific)
 239 supplemented with 10% fetal bovine serum (HyClone, Fisher
 240 Scientific) and 1% penicillin/streptomycin mix (Gibco, Fisher
 241 Scientific). A cell suspension (15 000 cells/cm²) was deposited in a
 242 droplet of supplemented α -MEM on top of the PDMS porous scaffold
 243 and incubated in an atmosphere containing 5% CO₂ at 37 °C for 1 h
 244 to enable the cells to adhere to the scaffold. Multiwell plates were then
 245 filled with additional supplemented α -MEM and incubated for 72 h.
 246 To prepare the sample for SEM characterization, cells were rinsed
 247 with PBS 1× (phosphate-buffered saline) solution and incubated in
 248 4% glutaraldehyde (Sigma) solution for 4 h at room temperature. The
 249 cells were then dehydrated by incubation in 50, 70, 90, and 100%
 250 ethanol solutions for 4 min at each step and dried for few hours at
 251 room temperature to remove alcohol residues. Immunofluorescence
 252 staining was performed as follows: cells were rinsed with PBS 1×,
 253 fixed with 10% formalin solution (Sigma) for 30 min, permeabilized in
 254 0.2% Triton X-100 for 3 min, and blocked in 3% BSA for 30 min. The
 255 samples were then incubated in phalloidine–rhodamine (Invitrogen,
 256 Fisher Scientific) at 1/200 dilution in PBS 1× for 30 min at 37 °C to
 257 stain the F-actin (protein of the cell cytoskeleton) and then in a DAPI
 258 (Thermo Scientific, Fisher Scientific) solution at 1/100 dilution in
 259 PBS 1× for 5 min at room temperature to stain the DNA in the
 260 nuclei. After staining, the cells were stored in PBS 1× solution at 4 °C.
 261 In an additional protocol for two-photon confocal imaging, the cells
 262 were rinsed with PBS 1×, stained with a mix of Hoechst (Invitrogen,
 263 Fisher Scientific) at 5 μ g/mL plus CMFDA (Invitrogen, Fisher
 264 Scientific) at 1/1000 dilution in DMEM without phenol red (Gibco,
 265 Fisher Scientific), incubated for 30 min at 37 °C, rinsed again with
 266 PBS 1×, and fixed for 30 min in 10% formalin solution (Sigma). The
 267 samples were stored in PBS 1× at 4 °C prior to imaging.
 268 Cytocompatibility was assessed by Live/Dead assay in the porous
 269 PDMS scaffold choosing flat PDMS and glass as control. All samples
 270 followed the protocol of plasma treatment and fibronectin coating
 271 mentioned above. After 72 h of culture in supplemented α -MEM, the
 272 samples were rinsed in PBS and cells were stained with calcein/
 273 ethidium (Live/Dead viability kit for mammalian cells, Fisher

Scientific) diluted in DMEM without phenol red for 30 min and 274
 then rinsed with the same medium for fluorescence characterization. 275

2.8. Immunofluorescence Characterization. Two different 276
 immunofluorescence imaging techniques were employed to investi- 277
 gate the cell distribution and proliferation taking place on the 3D 278
 PDMS scaffolds. 2D observations of the scaffolds' surface were 279
 performed using an Olympus C211 fluorescence microscope 280
 equipped with a X-Cite 120 Hg lamp, a BP (300–400 nm) filter for 281
 DAPI, a BP (575–595 nm) filter for calcein, a BP (518–573 nm) 282
 filter for phalloidin rhodamine/ethidium, and 5×, 10×, 20× and 50× 283
 objectives. 3D imaging of the scaffolds were performed using a two- 284
 photon confocal imaging system (AxioImager upright microscope 285
 LSM 7MP, Carl Zeiss). A pulsed femtosecond Ti:sapphire laser 286
 (Chameleon Ultra II; Coherent) tunable in the range of 690–1064 287
 nm was used as excitation light source. Z-stack acquisitions were 288
 performed with a 10× W-Plan Apochromat air objective with 0.45 289
 N.A. and a laser excitation wavelength tuned at 800 nm. An automatic 290
 z-compensation of the laser power was applied to provide a 291
 homogeneous imaging of the imaged volume of the 3D scaffold. 292
 Emitted light was detected through a descanned pathway leading to 293
 two nondescanned detectors and emission was recorded simulta- 294
 neously with two emission filters: a band pass (BP) filter, set at 500– 295
 550 nm (green channel, CMFDA), and a short-pass (SP) filter set at 296
 485 nm (blue channel, DAPI). Image processing and 3D 297
 reconstruction were performed by ImageJ and Imaris (Version 8.2, 298
 Bitplane) softwares. 299

3. RESULTS AND DISCUSSION

3.1. Morphological Characterization of the PDMS 300
Scaffolds. In Figure 2, we report how the tuning of the 301
 fundamental curing parameters (t_1 , along the vertical axis, and 302
 T_2 – P_2 , along the horizontal axis) affects the morphology of the 303
 PDMS porous scaffolds. The increment of the prereticulation 304
 process time affects the pore size and distribution as 305
 highlighted by comparing the rows of each column. The first 306
 row of Figure 2A–C, related to $t_1 = 30$ min, shows a typical 307

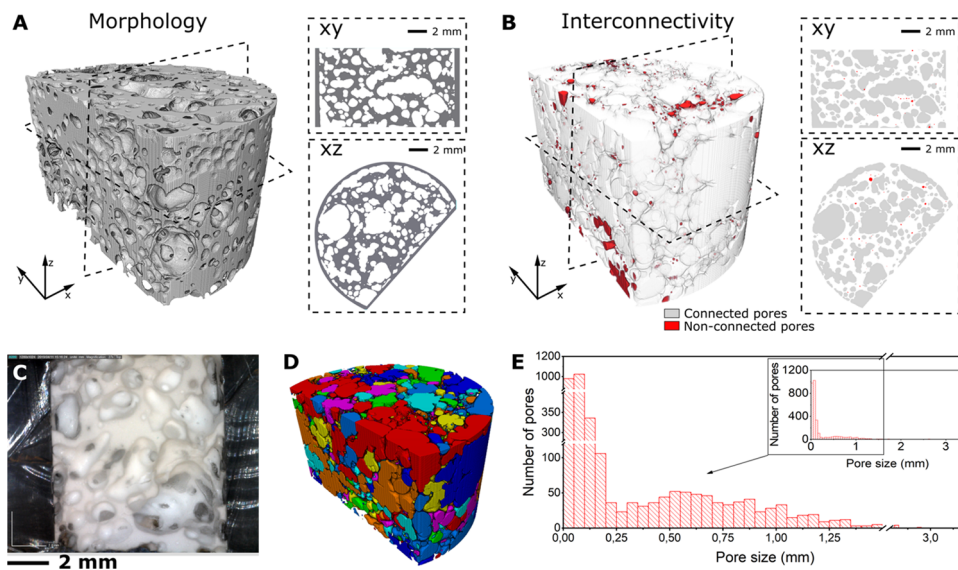


Figure 3. (A) 3D view of the PDMS scaffold after segmentation process. On the right, crosscuts of the reconstruction along the xy and xz planes. (B) 3D view of the pores: nonconnected pores (red), connected pores (white). On the right, crosscuts of the reconstruction along the xy and xz planes. (C) Optical micrograph of the inner core of a PDMS porous scaffold. (D) 3D colorimetric view of the pores size distribution with a watershed-based algorithm. (E) Distribution of the number of pores according to the pore size depicted in (D).

308 pore size of 1–3 mm with few submillimetric pores observable.
 309 When increasing t_1 up to 45 min, this pore size distribution
 310 slightly decreases (Figure 2D–F) down to 0.5–2 mm, while
 311 we observe at the same time an increased number of
 312 submillimetric pores. Finally, in the third row of Figure 2G–
 313 I, we report a reduction of the pore size coupled to an evident
 314 anisotropy of the pore orientation directed toward the open
 315 side of the PDMS scaffold holder.

316 A lower t_1 implies a less reticulated state of the PDMS within
 317 the emulsion when starting the final curing process. We then
 318 attribute the presence of large pore size (Figure 1A–C) to the
 319 easier expansion of the water steam in a less reticulated PDMS.
 320 We believe that this factor may induce the aggregation of
 321 submillimetric water bubbles and the consequent formation of
 322 larger cavities.

323 By comparing the first column (A, D, G) and second column
 324 (B, E, H) of Figure 2, we investigate the impact of the different
 325 temperatures T_2 (110 and 130 °C) employed in the second
 326 reticulation process. By fixing $t_1 = 30$ min, we cannot find
 327 striking differences in pore size in Figure 2A,B, while this
 328 difference becomes more evident for higher prereticulation
 329 periods, as reported in Figure 2D–F. In Figure 2G, we can
 330 observe strong anisotropy, with elongated pores whose main
 331 semiaxis is 2- to 3-fold larger than the minor semiaxis. The
 332 pores of Figure 2F show lower anisotropy compared to Figure
 333 2G but still much higher than the one of the pores in Figure
 334 2B,E. We suggest that the temperature of final reticulation (T_2)
 335 has a double impact on the reaction: on the one hand, it
 336 slightly modifies the speed of evaporation of the water bubbles;
 337 on the other hand, it varies the kinetics of the reticulation of
 338 the silicone. As we observe from the comparison of Figure
 339 2G,H, a lower temperature indeed tends to benefit the
 340 appearance of intrinsic anisotropy of the sample, which can be
 341 linked to a higher state of reticulation during the expansion of
 342 the water steam bubbles.

343 The third column (Figure 2C,F,I) explores the same
 344 temperature T_2 (130 °C) as in the second column (Figure
 345 2B,E,H) but with a different pressure P_2 (700 mbar instead of

400 mbar). By observing the samples obtained with a short
 346 prereticulation time t_1 (Figure 2B,C), it is possible to highlight
 347 the impact of the pressure over the pore size. While the sample
 348 2B features an average pore size of 1–3 mm, sample 2C
 349 presents a critical decrease of the typical size to 0.7–1.2 mm.
 350 For $t_1 = 45$ min, we observe the same tendency of reduced
 351 typical pore size within the PDMS porous scaffold, although
 352 this transition is less evident for the samples at 700 mbar
 353 (Figure 2C,F) than for the samples at 400 mbar (Figure 2B,E).
 354 Concerning the last row (Figure 2H,I), at long prereticulation
 355 time (60 min), we perceive a major reduction on the pore size
 356 coupled to a slight decrease in anisotropy of the pores. This
 357 behavior fits with the results obtained in the rest of the samples
 358 for those parameters.
 359

The general overview of Figure 2 shows then a progressive
 360 decrement of the pore size by increasing the time of
 361 prereticulation t_1 , which leads, at the same time, to an increase
 362 of the number of pores. We observe also an anisotropy
 363 tendency linked to the reticulation time (i.e., longer t_1
 364 increases the reticulation state of the silicone before the
 365 evaporation of the steam). This induces less motility in the
 366 emulsion that in turn leads to a further expansion of the
 367 bubbles in the direction of the apertures of the PDMS sample
 368 holder. Temperature T_2 , on the other hand, plays a dual role in
 369 the kinetics of the water evaporation and the kinetics of the
 370 reticulation process within the emulsion. Finally, pressure P_2
 371 hinders the expansion of the bubbles, limiting the final pore
 372 size. From a microscopic point of view, the PDMS porous
 373 scaffold presents a hierarchical porous structuration. A close-up
 374 view of the bulk material between the larger pores for different
 375 fabrication parameters (t_1 , T_2 , P_2) reveals a network of
 376 interconnected pores featuring smaller dimensions (5–30 μm)
 377 with openings of 1–5 μm (see Figure S2). This induces a
 378 roughness on the surface that might influence the perfusion
 379 and diffusion of culture medium with a global impact for the
 380 cell environment.
 381

To provide a more quantitative analysis of the pore size and
 382 spatial distribution, we performed a characterization of the
 383

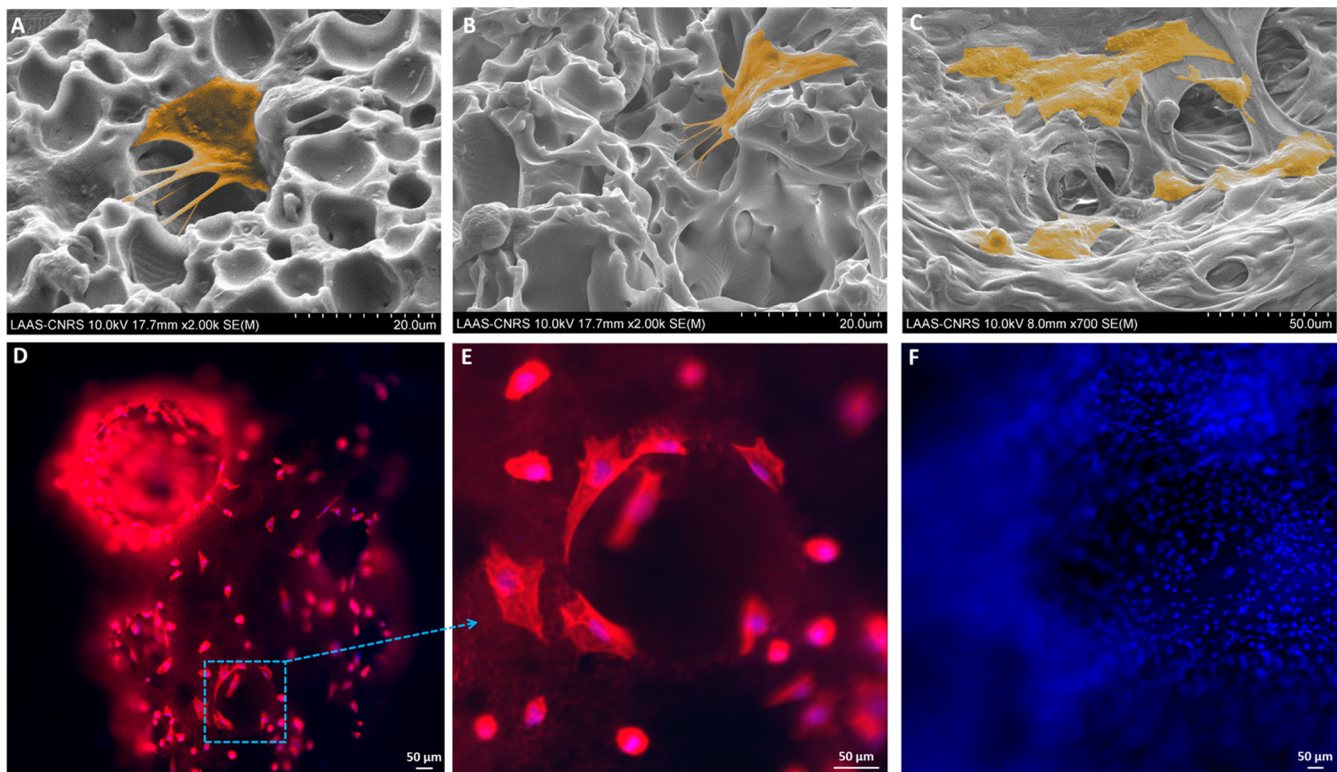


Figure 4. (A–C) False-colored SEM images highlighting the SaOS-2 cell morphology obtained on the PDMS porous scaffold; (D) immunofluorescence imaging of SaOS-2 cells colonizing the pores of the porous PDMS scaffold; (E) close-up view on the region enclosed in the blue dotted square in (D); (F) SaOS-2 “cellular-carpet” observed on the PDMS porous scaffold surface (red: phalloidin-F-actin; blue: DAPI-nuclei).

384 PDMS porous scaffold by employing X-ray tomography. The
 385 selected sample corresponds to the one of Figure 2B with $t_1 =$
 386 30 min, $T_2 = 130$, and $P_2 = 400$ mbar, which was then
 387 employed as 3D cell culture support.

388 Figure S3A shows a reconstructed slice of the sample, after
 389 denoising with a nonlocal means filter. A user-defined
 390 threshold was applied on each slice of the sample, leading to
 391 a stack of binarized images, as presented Figure S3B,
 392 corresponding to the slice shown in Figure S3A. The 3D
 393 reconstructed view of the PDMS porous scaffold in Figure 3A
 394 shows the porosity of the 3D architecture (whose optical
 395 micrograph is reported in Figure 3C). The crosscut views for
 396 the xy and xz planes show the inner core of the 3D
 397 architecture, proving a highly interconnected network with a
 398 wide spectrum of porosity. From those data, a porosity equal to
 399 around 57% was calculated from the binarized images (Figure
 400 S3C). A second validation of porosity using a watershed
 401 segmentation algorithm (for details, see Materials and
 402 Methods) was performed, resulting in a porosity equal to
 403 58.7%, as presented in Figure 3D. Connectivity of pores was
 404 also studied with the Axis Connectivity module available in
 405 Avizo. Figure 3B shows in red the nonconnected pores and in
 406 white the connected ones. The total volume of pores is equal
 407 to 406.2 mm^3 in the sample, and nonconnected pores
 408 represent only 4.3 mm^3 , corresponding to 1% of the total
 409 volume of pores. Nonconnected pores are either small pores
 410 $<100 \mu\text{m}$ in the matrix or pores that are localized in the walls
 411 of the cylindrical support.

412 Finally, pore size distribution was analyzed. The histogram
 413 plot in Figure 3E represents the distribution of pore equivalent
 414 diameter, whose colorimetric 3D visualization linking pore

diameters to different colors is reported in Figure 3D. The
 415 majority of the pores were reported to have a diameter
 416 between 0.02 and 0.10 mm (more detailed histograms are
 417 reported in Figure S4), although smaller pores (observed by
 418 SEM, Figure S2) could not be resolved due to resolution
 419 limitation ($18 \mu\text{m}$) of the X-ray microcomputed tomography
 420 setup (see Materials and Methods). These results were
 421 complemented with measurements of water retention
 422 performed over 48 h in the porous PDMS scaffold. As
 423 shown in Figure S5 and in the Supporting Video “Video_
 424 liquid_loading”, the percent of water remaining in the scaffold
 425 after soaking it in PBS for different periods of time increases
 426 until reaching a value close to $\sim 300\%$ (w:w). This value agrees
 427 with the high porosity and interconnectivity estimated by μCT .
 428

3.2. SEM and Immunofluorescence Characterization of SaOS-2 Cell Colonization of the PDMS Scaffolds.

429 To validate the compatibility of the developed PDMS architec-
 430 tures as a 3D cell culture tool, we tested the scaffold topology
 431 depicted in Figure 2B in the presence of SaOS-2 cells. Due to
 432 the morphology of the SaOS-2 cells and the topographical
 433 features of the PDMS porous scaffold, distinguishing the cells
 434 directly over a rugose surface is a laborious task. This is evident
 435 after comparing the morphologies of the osteosarcoma SaOS-2
 436 cell line on untreated PDMS flat surfaces (Figure S6A) and on
 437 plasma treated/fibronectin-coated PDMS surfaces (Figure
 438 S6B). While on untreated surfaces we observe the same ratio
 439 of cells holding the expected flattened morphology and the less
 440 conventional globular one, on the treated surfaces we report
 441 only the flattened cytoskeletal configuration coupled to a more
 442 marked expression of typical round protrusions already
 443 observed elsewhere.⁵⁵ In such context, the role of roughness
 444

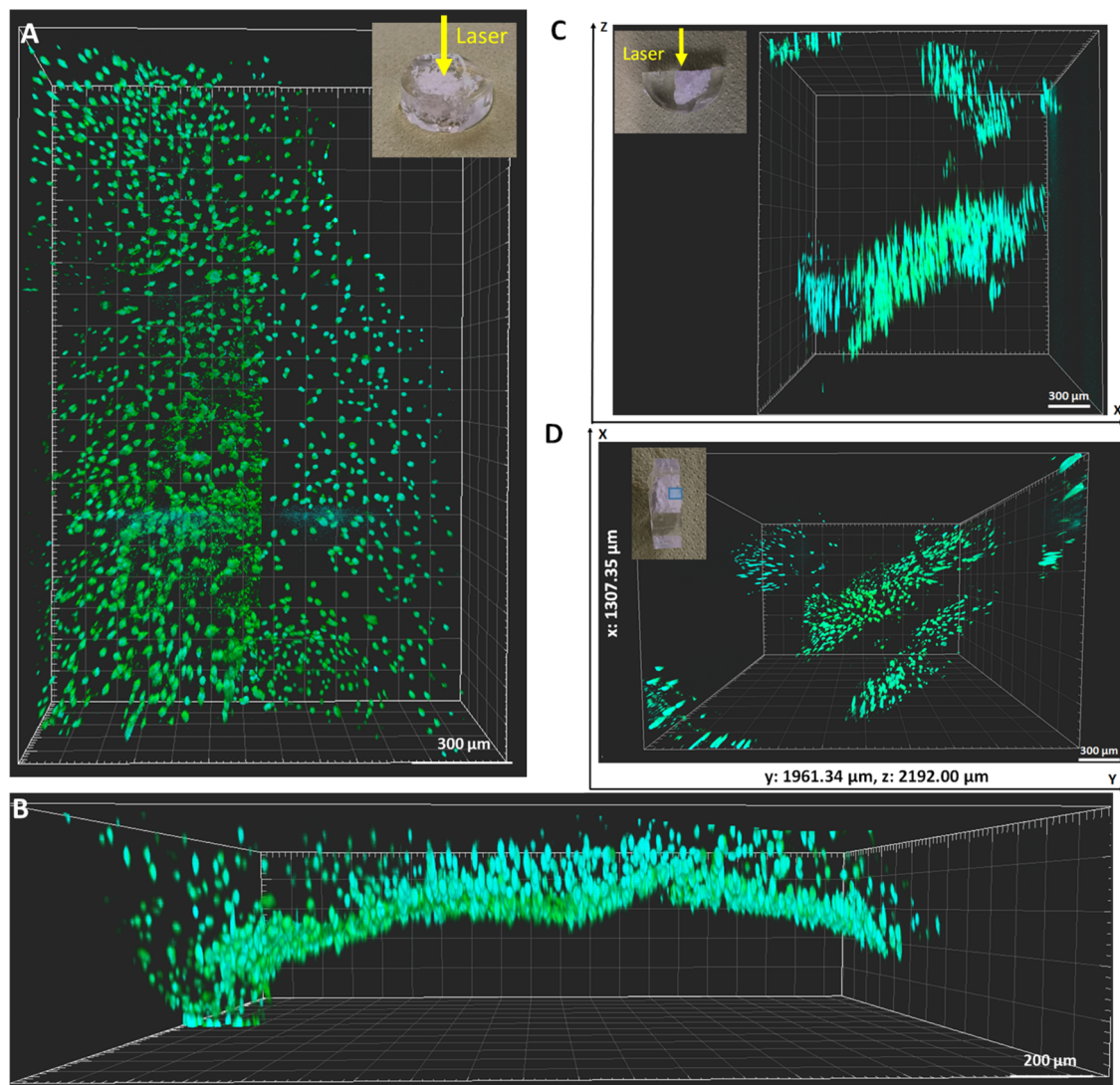


Figure 5. Two-photon confocal imaging of the PDMS porous scaffold colonized by SaOS-2 cells. (A) *xy* view of the 3D reconstruction of the scaffold imaged with the laser beam impinging the sample as shown in the inset; (B) *xz* view of the 3D reconstruction in (A); (C) *xz* view of the 3D reconstruction of a cross-cut of the scaffold imaged with the laser beam impinging the sample as shown in the inset; (D) *xy* view of the 3D reconstruction in (C) (the inset shows the sample orientation and the region depicted in the 3D reconstruction (green: CMFDA; blue: DAPI-nuclei)).

446 and surface chemistry regulating cell adhesion mechanisms has
447 been previously observed for nonpolymeric surfaces.^{56,57}

448 In addition to its role in the adsorption of adhesion proteins,
449 plasma treatments are indeed known to induce the presence of
450 nanotopography features and surface chemistry changes, which
451 are reflected in an augmentation of oxygen content and an
452 enhancement of surface energy associated with an increase in
453 wettability that influences cell adhesion on polymeric
454 surfaces.^{58,59} Concerning the distribution and welfare of cells
455 within the PDMS porous scaffold, in Figure 4A–C, we present
456 false-colored SEM close-up images highlighting the typical cell
457 morphology that we observed in different regions of the
458 scaffold. SaOS-2 cells tend to develop cytoskeletal extensions
459 anchoring to the side walls of the micropores in a
460 tridimensional spatial configuration, as observed in Figure
461 4A,B. The evidence of the flattened elongated morphology of a
462 cluster of cells in other regions of the scaffold characterized by
463 PDMS meshes is shown in Figure 4C. Furthermore, we can
464 discriminate several round protuberances within the cellular

cytoskeleton that we associate with regular features of
465 mineralized buds and calcospherulites related to the process
466 of mineral deposition of this cell line.^{60,61} This behavior could
467 be associated with a predifferentiation stage, although a deeper
468 evaluation of differentiation markers is required to confirm this
469 assessment. Prospective analysis of the calcification process by
470 red alizarin staining will be carried out in the future to validate
471 this conclusion. Furthermore, cell viability test, shown in
472 Figure S7, was addressed by Live/Dead assay on the porous
473 PDMS scaffold against two positive controls, flat PDMS and
474 glass. Cell viability reached ~99% of the cell population over
475 the porous PDMS scaffold. This result compares with the
476 performance of both positive controls showing as well a cell
477 viability of ~99% and a more homogeneous cell distribution.
478

To better visualize typical morphological features of the
479 SaOS cells cultured on the 3D scaffold, in Figure 4D–F, we
480 report the epifluorescence characterization of the osteosarcoma
481 cells over the PDMS porous scaffold. In Figure 4D, we can
482 observe the cell spreading and colonization of different pores,
483

484 where the F-actin, the main component of microfilaments in
485 the cytoskeleton, is stained in red with phalloidin/rhodamine
486 while the nuclei are stained in blue with DAPI. We can
487 distinguish several cells colonizing the full structure at different
488 out-focus planes. In Figure 4E, we show the close-up view of a
489 single pore (enclosed by the blue square in Figure 4D)
490 depicting how the elongated cytoskeleton of the cells adapts to
491 the surrounding environments by following the geometrical
492 profile of the pore. Finally, in Figure 4F, we present a
493 fluorescence image of another region of the scaffold where we
494 can easily identify cell nuclei and observe how cells cover
495 homogeneously the whole surface of the PDMS porous
496 scaffold.

497 To get a clear view of the 3D cell colonization scenario of
498 the PDMS porous scaffold, in Figure 5, we report several 3D
499 reconstructions obtained via two-photon confocal imaging, a
500 widely used technique for unveiling cell features in the inner
501 core of 3D architectures otherwise not accessible by more
502 conventional morphological imaging approaches (e.g., SEM
503 and AFM).^{33,35} In Figure 5A,B, we report the characterization
504 of the upper part of the 3D scaffold (i.e., the one on which the
505 cell medium containing the SaOS-2 cells was deposited),
506 impinged by the laser beam as shown in the inset of Figure 5A,
507 where we highlighted the nuclear marker DAPI (in blue) and
508 the CMFDA one (in green), which is able to stain the whole
509 cytoplasm of the cells (Figure S8). The overall field of view of
510 the acquisition is $1.96 \times 1.30 \times 0.49 \text{ mm}^3$ (obtained by
511 employing a mosaic modality where we imaged smaller areas
512 and then stitched them together). Figure 5A shows a quite
513 homogeneous cell coverage of the PDMS scaffold surface,
514 while Figure 5B highlights how cells are able to colonize the
515 inner part of the architecture by infiltrating open pores (such
516 as the one on the left-hand side of the figure). We attribute the
517 absence of cells in the central part of the 3D reconstruction
518 either to the small dimensions of the pores (compared to the
519 SaOS-2 cell size) in that region or, more likely, to the fact that
520 denser regions of PDMS (becoming opaque during the
521 fabrication because of the trapping of air bubbles) hinder the
522 passage of photons. A clearer overview of the 3D imaging
523 acquisition can be seen in the Supporting Video “Video_
524 top_surface”. To have a further insight into the cellular
525 distribution within the 3D scaffold, we performed also a cross-
526 cut on it by using a surgical blade to reveal the inner part of the
527 structure. Figure 5C,D shows, respectively, the x , z and x , y
528 views of a $1.30 \times 1.96 \times 2.19 \text{ mm}^3$ region of the PDMS porous
529 scaffold (always by employing the mosaic modality mentioned
530 above) with the laser impinging this time the inner surface of
531 the cross-cut, as depicted in the inset of Figure 5C. The two
532 different views show the presence of omnidirectional cell
533 clusters infiltrating both the superficial layers of the scaffold
534 and the inner areas down to a depth $\approx 2.2 \text{ mm}$ along the z axis
535 and $\approx 2 \text{ mm}$ along the y axis. A high-resolution video
536 (“Video_cross_cut”) of the slices composing the 3D
537 reconstruction is available in the Supporting Information.

4. CONCLUSIONS

538 In this work, we reported a novel 3D structuration technique
539 that provides a fast and cheap process to fabricate porous
540 scaffolds made of a widely used biocompatible silicone such as
541 PDMS. This technique involves a simple combination of water
542 and silicone to form an emulsion that is further transformed
543 into a highly porous scaffold using a two-step reticulation
544 process. The fabrication takes place under temperature-/

pressure-controlled environment that could be easily scalable
545 for mass production. Scanning electron microscopy character-
546 ization provided a deep understanding of the different achieved
547 morphologies confirming a control of the porous distribution
548 associated with a hierarchical macro-micro structuration of the
549 available surface that could have an impact on the biological
550 perfusion and diffusion of nourishment. 3D morphological
551 characterization was carried out by X-ray computed tomog-
552 raphy and allowed to evaluate the general porosity and the size
553 distribution of the pores as well as to investigate the
554 interconnectivity of the PDMS porous scaffold. The format
555 chosen to evaluate the performance of cell culture presented a
556 general porosity of $\sim 60\%$ with a $\sim 98\%$ global interconnectiv-
557 ity. Osteosarcoma SaOS-2 cells were seeded on the PDMS
558 porous scaffold to evaluate the efficiency of the cell
559 colonization and perfusion. SEM investigation in combination
560 with fluorescence immune-staining imaging demonstrated a
561 high level of cell adhesion and some features of advanced cell
562 maturation. Two-photon confocal reconstruction displayed
563 information about the cell colonization taking place within
564 volumes of several mm^3 in the inner core of the scaffold,
565 proving a homogeneous tridimensional distribution of cells and
566 their proliferation through all of the available pores.

As the fabrication process is simple, adapted with injection
568 techniques, and amenable to large-scale production, we believe
569 that it will open intriguing opportunities aiming at the
570 integration of 3D porous scaffolds in bioreactors or micro-
571 physiological systems. Further studies will be devoted to the
572 impact of the scaffold porosity and architecture on inner flow
573 patterns provided by microfluidic systems and subsequent
574 studies on cell proliferation and differentiation.

5 Supporting Information

3D model of the 3D-printed mold employed for the
580 fabrication of the PDMS sample holder; SEM close-up
581 images of the PDMS nest scaffold for different
582 reticulation conditions; X-ray tomography analysis,
583 porosity, and pore diameter distribution; and SEM and
584 immunofluorescence characterization of SaOS-2 cells
(PDF)

Video_top_surface (MP4)

Video_cross_cut (MP4)

Video_liquid_loading (MP4)

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