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1 Sacrificial Mold-Assisted 3D Printing of Stable 2 Biocompatible Gelatin Scaffolds

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15

16 **Abstract:** Gelatin is a fascinating biomaterial due to its biocompatibility, biodegradability and
17 chemical composition. The engineering of gelatin scaffolds with the most appropriate architecture
18 is crucial for tissue engineering applications. Currently available porogen assisted scaffold
19 fabrication technique are limited for the fabrication of repeatable and customized scaffolds. Three
20 dimensional printing is an appropriate technique to prepare customized structure. In this report,
21 customized gelatin scaffolds were fabricated using 3D printed polyvinyl alcohol (PVA) sacrificial
22 molds. Gelatin was stabilized through crosslinking and the PVA was solubilized to obtain 3D
23 customized gelatin scaffolds. The gelatin scaffolds displayed tunable pore size from 350 to 1200 μm .
24 The influence of PVA infill density on the gelatin scaffold porous structure and interconnectivity
25 was also analyzed. Furthermore, the gelatin scaffold stability in phosphate buffered saline solution
26 and their swelling capacity demonstrated that they were effectively crosslinked. The scaffold
27 cytocompatibility and the good cell attachment and proliferation on these scaffolds showed that the
28 sacrificial mold-assisted 3D printing is suitable for producing customizable gelatin scaffolds for
29 biomedical applications.

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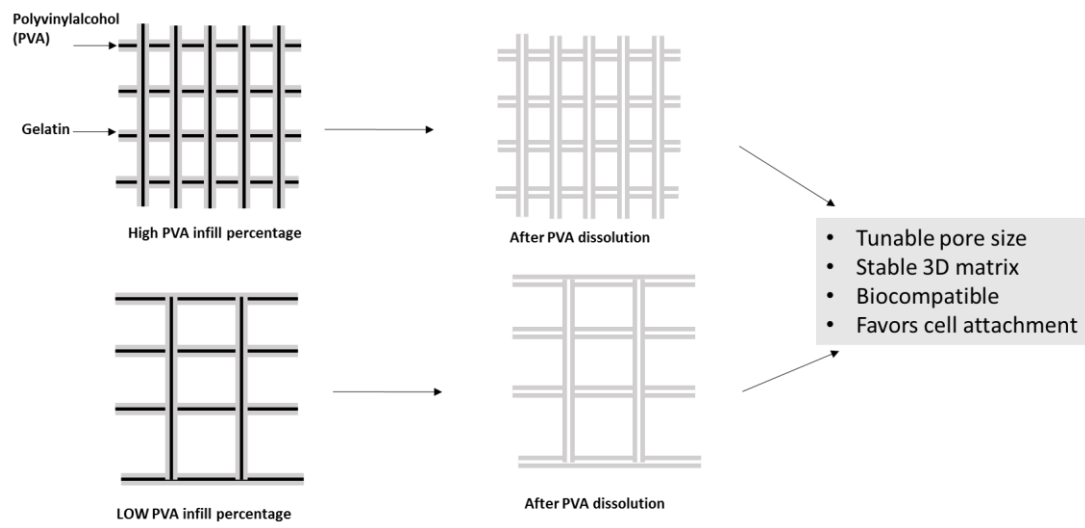
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34 **Graphical Abstract**

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37 Keywords: 3D printing; fused deposition modeling; gelatin; sacrificial mold; polyvinyl alcohol

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40 1. Introduction

41 The major shortcoming of two-dimensional (2D) cell culture models is their inability to emulate
42 the 3D cell environment of tissues [1, 2]. Conversely, 3D cell culture approaches almost recapitulates
43 the complex mechanical and chemical environment of tissues, and this is crucial for drug screening
44 and tissue engineering [3]. Polymer-based scaffolds also mimic the 3D cell culture environment. For
45 instance, scaffolds fabricated with synthetic or bio-based polymers are often used as templates for
46 tissue regeneration. However, 3D engineering methods can be advantageously employed to fabricate
47 scaffolds that allow cell infiltration and nutrient transport.

48 Scaffolds have been fabricated using various techniques, such as particulate leaching, freeze-
49 drying, phase separation, gas foaming and electrospinning [4], that permit the formation of
50 interconnected porous structures and tunable pore size. However, it is difficult to fabricate
51 reproducible structures and to control the interconnected porosity [4]. This issue could be solved by
52 selecting 3D printing technologies [5, 6], representing advantageous tools for the production of
53 reproducible and controlled structures. 3D printed sacrificial molds or sacrificial mold-assisted
54 techniques have been widely used for manufacturing microfluidic channels, polymeric scaffolds,
55 inorganic 3D matrix materials, and microneedles [7-10].

56 Gelatin and collagen are highly attractive biopolymers for scaffold production because they are
57 inherently biocompatible and biodegradable. They also contain the Arg-Gly-Asp (RGD) motif and
58 matrix metallo protease degradable molecule that can facilitate the cell-scaffold interaction. 3D
59 printing of gelatin using the fused deposition modeling (FDM) technique is arduous due to the lack
60 of sharp melting temperature of gelatin. Gelatin has been employed for ink-based 3D printing [11],
61 which requires surface modification of gelatin [12]. Unmodified gelatin is printable below ambient
62 temperature but additional crosslinking after the printing is mandatory to stabilize the 3D scaffolds.
63 However, the porous structure is obtained only through the inter filament gap in extrusion 3D
64 printing. Interestingly, dissolution of the sacrificial mold results in interconnected porous gelatin
65 scaffolds by connecting multiple layers through hollow tubes.

66 On the other hand, gelatin scaffolds could be easily fabricated using polyvinyl alcohol (PVA)
67 sacrificial molds printed using the FDM technique. PVA is a water-soluble thermoplastic suitable for
68 the 3D printing of complex structures with high degree of control using the thermal extrusion method
69 [8]. It has been widely used to produce sacrificial molds for the fabrication of poly(dimethyl siloxane)
70 scaffolds [7, 8, 13], but not of 3D biopolymer scaffolds. Li et al. attempted to embed 3D-printed PVA
71 filaments in a gelatin matrix to obtain perfusable vascular-like structures upon PVA dissolution [14].
72 However, fabrication of self-standing interconnected porous gelatin scaffolds with controlled
73 architecture has not been reported previously. In a recent work, Jung et al. described photocurable
74 and alkaline soluble sacrificial molds for the fabrication of gelatin/polycaprolactone 3D scaffolds.

75 They used sodium hydroxide for the dissolution of the sacrificial mold, and they often observed
76 permanent deformation of the scaffolds. Moreover, the fabrication of gelatin scaffolds was difficult
77 [15]. Therefore, developing an appropriate method to fabricate customized gelatin scaffolds is crucial.

78 Besides PVA, various other materials are employed for sacrificial mold fabrication. For example,
79 perfusable complex vascular architectures were 3D printed using water-soluble sugar glass as
80 sacrificial mold and gelled polydimethylsiloxane. Dissolution of the sacrificial mold led to a self-
81 standing structure that could be perfused [16]. Similarly, monolithic microfluidic channels were
82 fabricated using 3D printed water-soluble sugar-based sacrificial molds [17]. However, these studies
83 did not investigate the fabrication of biopolymer-based interconnected porous scaffolds. Thermo-
84 responsive polymers also have been used as sacrificial molds. For instance, poloxamer 407 (Pluronic
85 F127) displays characteristic properties of gel to sol transformation upon cooling that favor the easy
86 removal of the sacrificial mold [18].

87 In this report, a sacrificial mold-assisted 3D printing method was used to fabricate self-standing
88 water-insoluble gelatin scaffolds with tunable pore size and porosity. The PVA-based sacrificial
89 molds were 3D printed using the FDM technique and different polymer infill densities. The influence
90 of PVA infill density on the gelatin scaffold pore size was evaluated, followed by analysis of the
91 gelatin scaffold swelling capacity in phosphate buffered saline (PBS). In addition, the gelatin scaffold
92 biocompatibility and cell attachment were studied using cell staining and scanning electron
93 microscopy (SEM).

94

95 **2. Materials and Methods**

96 Gelatin (9000-70-8), glutaraldehyde (25%, 111-30-8), absolute ethanol, glacial acetic acid (100%,
97 64-19-7), hexamethyldisilazane ($\geq 99\%$, 999-97-3), PBS, DMSO and calcein-AM solution were
98 purchased from Merck and used as received. The cell culture reagents were MEM alpha medium,
99 penicillin/streptomycin and trypsin-EDTA (all from Gibco) and fetal bovine serum (Eurobio). PVA
100 filament was from Makershop.

101 *2.1. 3D printing of sacrificial PVA molds*

102 Sacrificial PVA molds were fabricated using PVA filament and a Prusa Research MK2S 3D
103 printer (see Table 1 for the 3D printing parameters). Computer-aided design software (Design Spark
104 Mechanical) was used for drawing the PVA molds and the STL file was generated using the
105 Prusa3Dslicer software. The final dimensions of the PVA sacrificial mold are 10 mm length, 10 mm
106 depth and 6 mm height. Each batch of PVA molds requires 8 minutes to be printed.

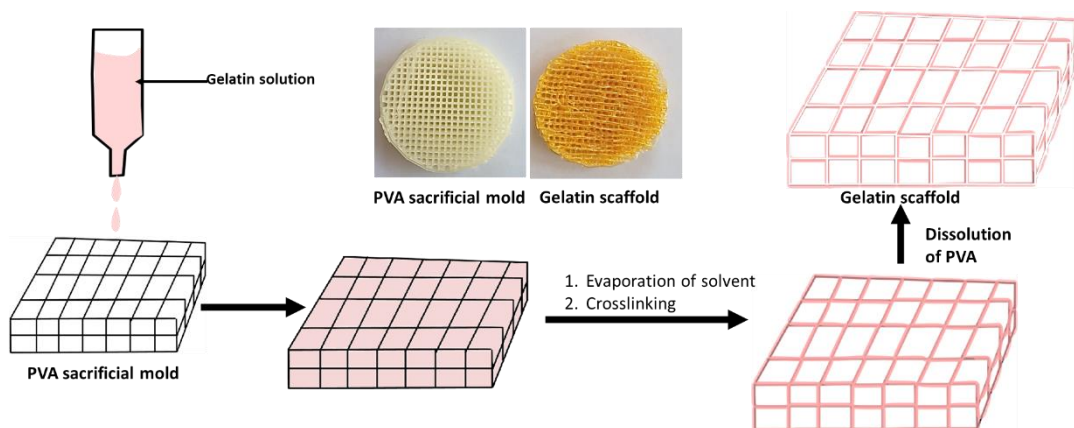
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Table 1. 3D printing parameters.

| Parameters | Value |
|-----------------------------------|-------------|
| First layer height | 0.1 mm |
| Other layers' height | 0.2 mm |
| Perimeters | 1 |
| Number of top & bottom layers | 0 & 5 |
| Infill percentage | 40% and 55% |
| Combine infill layers | 3 |
| Infill angle | 45° |
| Bed temperature | 60 °C |
| Nozzle temperature (1st layer) | 215 °C |
| Nozzle temperature (other layers) | 210 °C |

108 2.2. Fabrication of 3D gelatin scaffolds

109 The gelatin solution (10% w/v) was prepared at 60 °C using glacial acetic acid as solvent. The 3D
110 PVA molds fabricated with 40% and 55% of PVA infill density were denoted as PVA40 and PVA55,
111 respectively. Then, the gelatin solution (100 μ L/PVA mold) was poured and let to fill the PVA molds.
112 PVA molds filled with the gelatin solution were dried at room temperature for 24 hours, and then
113 crosslinked using 0.5% glutaraldehyde solution (3 mL/4 molds) for 24 hours. The glutaraldehyde
114 solution was prepared from 25% (v/v) glutaraldehyde stock solution using absolute ethanol. Finally,
115 the crosslinked gelatin-filled PVA molds were immersed in water at 50°C, and this step was repeated
116 five times to completely dissolve the mold. PVA dissolution resulted in water-insoluble gelatin
117 scaffolds. Gelatin scaffolds fabricated using PVA40 and PVA55 sacrificial molds were denoted as G40
118 and G55, respectively (Figure 1).



119

120

Figure 1. Schematic representation of gelatin scaffold fabrication.

121 2.3. Gelatin scaffold characterization

122 The morphology of the PVA sacrificial molds and gelatin scaffolds were analyzed by SEM
123 (HITACHI S4800) after coating with platinum using an ion sputter coater. The 3D architecture of the
124 scaffolds was analyzed using EasyTom XL 150. The gelatin scaffold swelling ratio was calculated by
125 equilibrating pre-weighed gelatin scaffolds in 0.1 M PBS at pH 7.4 at 37 °C. At different time points,
126 equilibrated samples were removed and the weight of the scaffolds was recorded. The scaffolds were
127 carefully put back in PBS after weight recording.

128 The swelling ratio was calculated with the following equation (1):

$$\text{Swelling Ratio} = \frac{Wt - W_0}{W_0} \times 100 \quad (1)$$

129 where W_t is the gelatin scaffold weight at the 't' time, and W_0 is the gelatin scaffold initial weight.

130 Fourier-transform infrared spectroscopy (FTIR) spectra of gelatin scaffolds were recorded at 600
131 to 4000 cm^{-1} frequency and 4 cm^{-1} resolution using a NEXUS instrument, equipped with an attenuated
132 total reflection accessory.

133 2.4. Cell viability assays

134 MG63 osteosarcoma cell line (obtained from ATCC) were cultured in MEM alpha medium
135 supplemented with 1% penicillin/streptomycin (Gibco 15140-122) and 10% fetal bovine serum
136 (Eurobio CVFSVF00-01) at 37 °C in 5% CO_2 . Cells were maintained in 10 cm diameter dishes and
137 0.05% trypsin-EDTA (Gibco 25300-054) was used for passaging. Gelatin scaffolds were sterilized with
138 60% ethanol (w/v) and rinsed in PBS. For cell viability assays, 1.5×10^3 MG63 cells/well were plated in
139 96-well plates and let to attach for 24 hours. Then, the sterilized gelatin scaffold was put on top of the
140 cell layer and cell viability was assessed with the MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl
141 tetrazolium bromide) assay at 1, 4 and 8 days (n=3). Briefly, cells were incubated with 0.05 mg mL^{-1}
142 of MTT solution (100 μL /well) for 2h to allow the formation of purple-colored formazan crystals.
143 Then, formazan crystals were dissolved by adding 100 μL /well of dimethyl sulfoxide (BDH Prolab
144 23486.297), and absorbance was recorded at 560 nm using a Multiskan plate reader (Thermo
145 Scientific, USA).

146 2.5. Calcein staining to monitor cell attachment and proliferation

147 MG63 cells (5×10^3 cells/scaffold) were cultured on gelatin scaffolds for 4 days. Then, they were
148 incubated with the calcein-AM solution (maker) at 37 °C in 5% CO_2 for 30 minutes and monitored by
149 epifluorescence microscopy (DM6000 Leica). SEM was also used to characterize the cells attached on
150 the scaffolds. Briefly, cells cultured on scaffolds were fixed with 2% glutaraldehyde solution for 1
151 hour and then scaffolds were washed with PBS. Cells attached to the scaffolds were incubated with

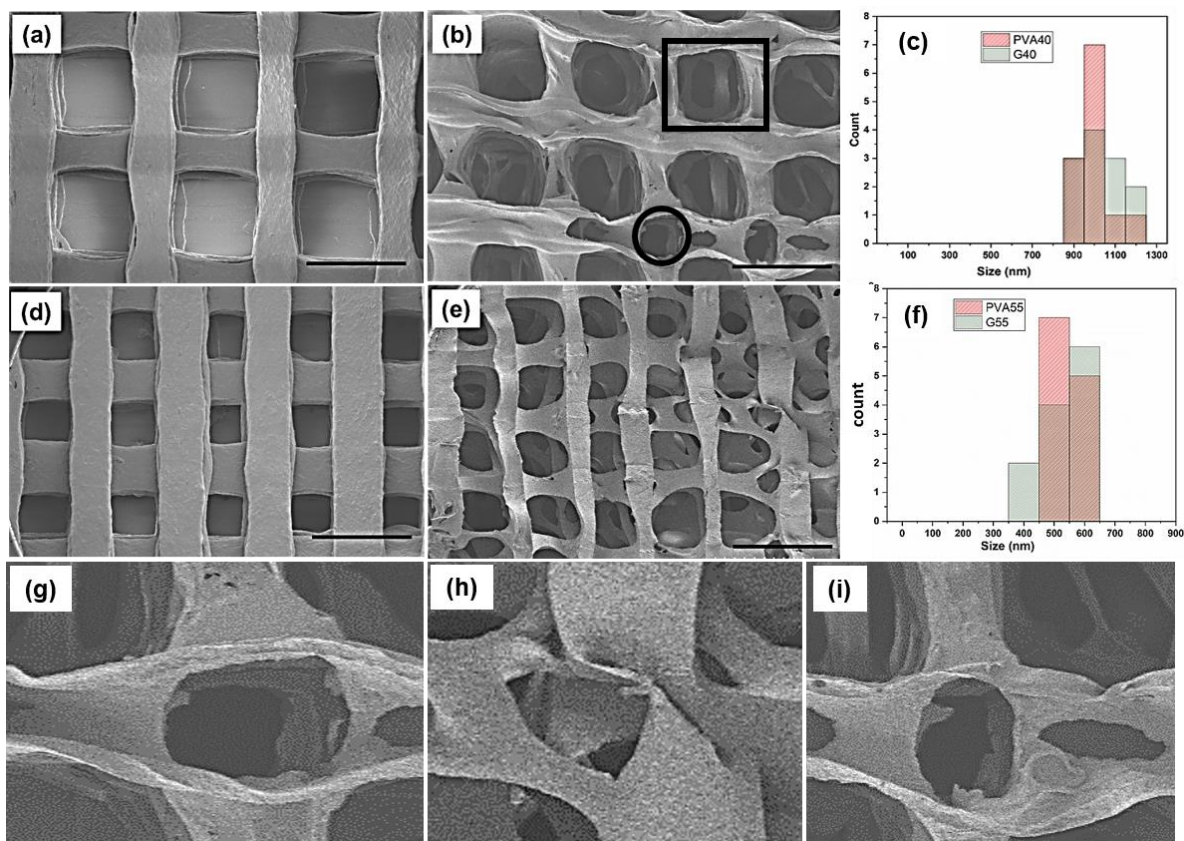
152 increasing ethanol concentrations (10%, 20%, 40%, 70% and 90% (v/v)), followed by drying using
153 hexamethyldisilazane.

154

155 **3. Results and Discussion**

156 Gelatin scaffolds were 3D printed using PVA sacrificial molds with a porous structure that were
157 then dissolved in water. Designing scaffolds with appropriate pore size and properties allowing cell
158 adhesion and proliferation is essential for their use in tissue engineering. For instance, Murphy et al.
159 reported that high pore size ($> 300 \mu\text{m}$) facilitates cell infiltration and proliferation, vascularization
160 and bone ingrowth [19]. Therefore, to obtain tunable pore size, two different PVA molds with
161 different polymer infill density (40% and 55%) were used (PVA40 and PVA55, respectively). The
162 morphology of the PVA molds (Figure 2a and d) and gelatin scaffolds (Figure 2b and e) was analyzed
163 by SEM. The gelatin scaffolds displayed a tunable pore size ranging from 350 to 1200 μm (Figure 2c
164 and f) which shows that the gelatin scaffolds are suitable to facilitate cell infiltration. Although gelatin
165 is highly water soluble, the crosslinked gelatin scaffolds maintained the architecture and porous
166 morphology even after dissolution of the PVA sacrificial molds, highlighting their stability in
167 aqueous solution. Sacrificial PVA molds generate the porous structure to the gelatin scaffolds in three
168 ways: I. dissolution of sacrificial mold filament leads to the hollow filament of gelatin, II. Inter gelatin
169 filament gap and III. Dissolution of sacrificial filament junction supports to connect adjacent layers
170 of gelatin filaments. Such interconnectivities are crucial for cell migration and infiltration into
171 multiple layers of scaffolds.

172 The architecture of the gelatin scaffolds was the replica of the PVA sacrificial molds and the
173 number of pores per unit area in each scaffold (G40 and G55) correlated with their number in the
174 corresponding sacrificial mold (PVA40 and PVA55). However, the pore number in any given area
175 was higher in the gelatin scaffolds than in the PVA sacrificial molds, due to the shrinkage of the
176 gelatin scaffold during drying (Figure 2a to f).

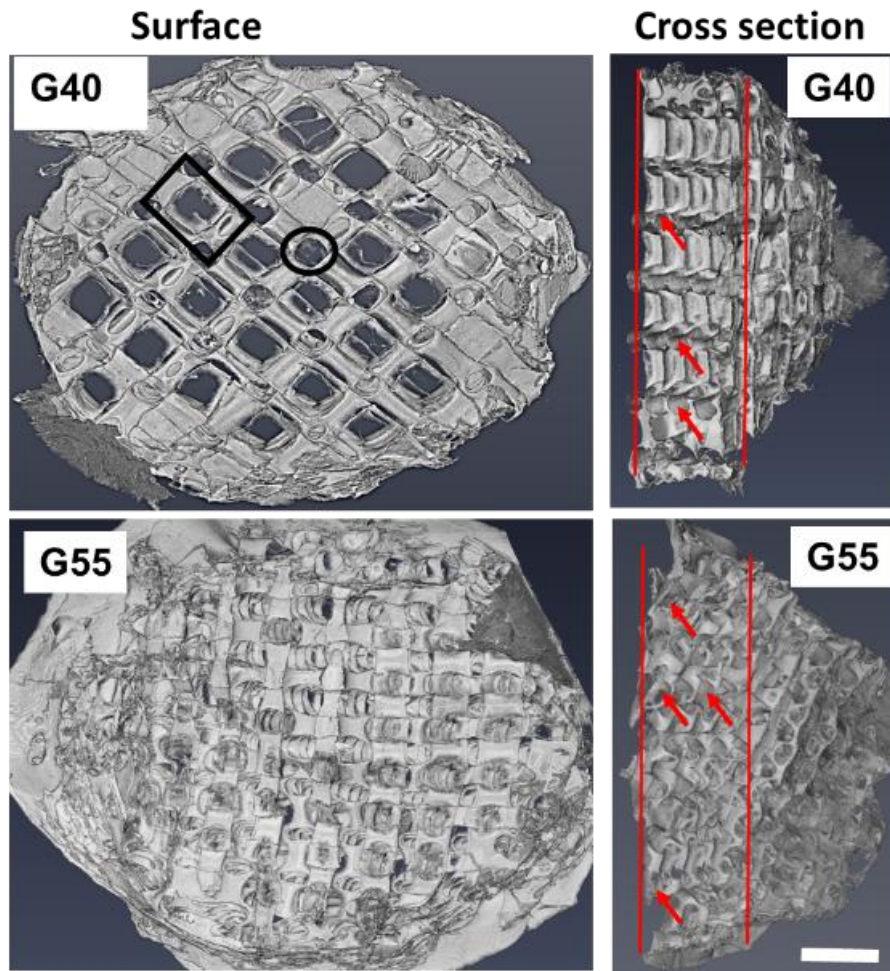


177
 178 **Figure 2.** SEM images of PVA40 (a), G40 (b,g,i), PVA55 (d), G55 (e,h), and pore size distribution in sacrificial
 179 PVA molds and gelatin scaffolds (c and f).

180 Analysis of the 3D architecture of the G40 and G55 scaffolds by X-ray microtomography (Figure
 181 3) showed that both gelatin scaffold types had a porous structure imparted by the porous structure
 182 of the PVA sacrificial molds (see PVA40 and PVA55 images in Figure 2a and 2d). This type of pore is
 183 indicated by black squares in Figures 2 and 3a. Moreover, a second pore type was formed after PVA
 184 dissolution (black circles in Figure 2 and 3a). Indeed, the gelatin deposited and crosslinked around
 185 the PVA matrix during scaffold synthesis formed gelatin tubes after PVA dissolution (Figures 2g to
 186 i). The microtomography images of G40 and G55 scaffolds showed that gelatin uniformly diffused in
 187 the two types of sacrificial molds (Figure 3). It shows that preparation of gelatin solution in glacial
 188 acetic acid prevented the dissolution of sacrificial mold before crosslinking of gelatin. Further, high
 189 solubility of PVA in water favors the gelatin scaffolds formation. As observed also on the SEM
 190 images, the gelatin scaffold pores were strongly influenced by the PVA sacrificial mold porosity
 191 (compare Figure 3a and c). The two pore types (inter- and intra-gelatin tubes, indicated by the square
 192 and circle, respectively, in Figure 3) were clearly larger in G40 than in G55 scaffolds.

193 Moreover, the two scaffolds presented a 3D interconnected porous structure (Figure 3b and d). The
 194 tubes formed after dissolution of the PVA sacrificial molds were observed along the rows and
 195 columns and introduced a high interconnectivity within the scaffolds. The PVA sacrificial mold

196 were 3D printed in such a way that the two adjacent layers were separated by a specific distance.
197 This helped to interconnect the porous structures formed between gelatin tubes. Presence of these
198 interconnected porous structures is crucial to facilitate cell infiltration.



199

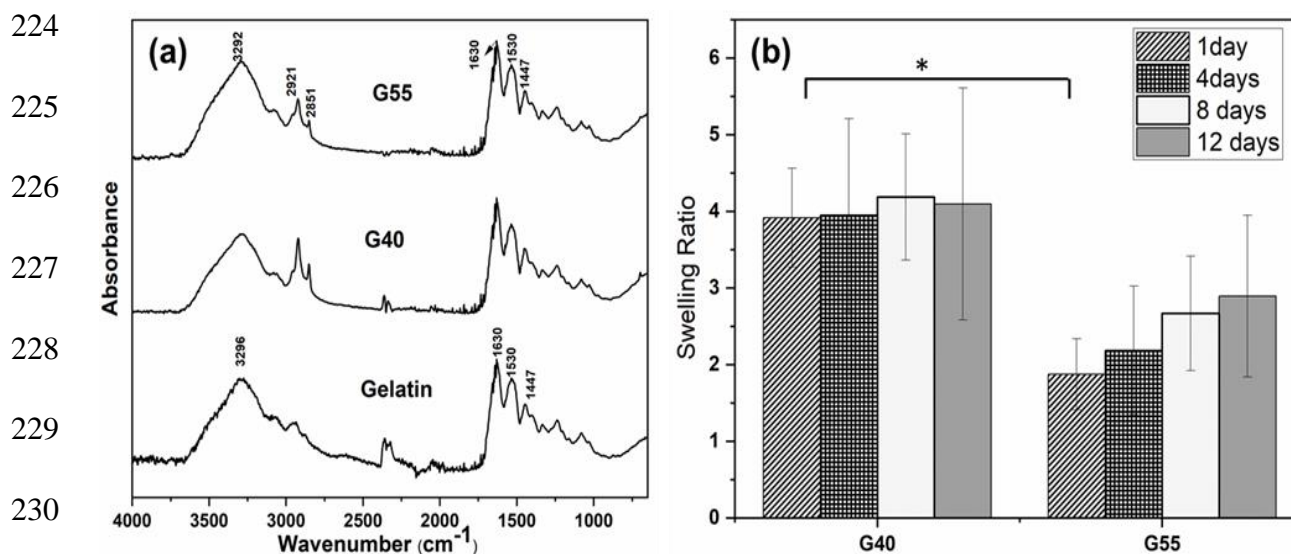
200 **Figure 3.** X-ray microtomography images of gelatin scaffolds (scale bar is 2 mm). The cross-sectional
201 area is delineated by the red lines; arrows indicates the porous structure.

202 FTIR spectra of the G40 and G55 gelatin scaffolds (Figure 4a) showed, as expected [20], the
203 presence of peaks characteristic of gelatin (amide I and amide II at 1650 cm^{-1} and 1539 cm^{-1} ,
204 respectively). C-H stretching vibration of the alkyl group of PVA was also observed at 2921 cm^{-1} and
205 2851 cm^{-1} [21]. Although SEM images (Figure 2) clearly confirmed PVA dissolution and the formation
206 of gelatin tubes, FTIR indicated that some PVA molecules were still attached to the gelatin scaffolds.
207 The primary amine groups in gelatin are responsible for the crosslinking. Conversely, the hydroxyl
208 groups of PVA react less with glutaraldehyde compared with the gelatin amine groups. Many studies
209 reported that PVA crosslinking is an acid-catalyzed reaction and that it requires high glutaraldehyde
210 concentrations [22, 23]. Here, crosslinking was performed with a high concentration of
211 glutaraldehyde (0.5% solution), which might explain the fact that traces of PVA are covalently

212 attached to gelatin. Decreasing the glutaraldehyde concentration could diminish the PVA attachment
213 on the gelatin scaffolds.

214 Analysis of the gelatin scaffold swelling capacity showed that G40 scaffolds reached the swelling
215 equilibrium after 1 day in PBS (Figure 4b). By contrast, the G55 scaffold exhibited a significantly lower
216 swelling ratio (approximately 2-fold less as compared to the G40 scaffold) after 1 day in PBS (Figure
217 4b). Interestingly, the swelling capacity of the G55 scaffold increased upon time (increase of the
218 swelling ratio up to 12 days in PBS). This delay in the swelling capacity of the G55 scaffold can be
219 explained due to the increased degree of crosslinking compare to G40. Of note, both the G40 and G55
220 scaffolds did not exhibit significant weight loss even after 12 days in PBS and remained easily
221 manipulable (Figure 4b), thus indicating that they are insoluble and validating their stability in
222 solution.

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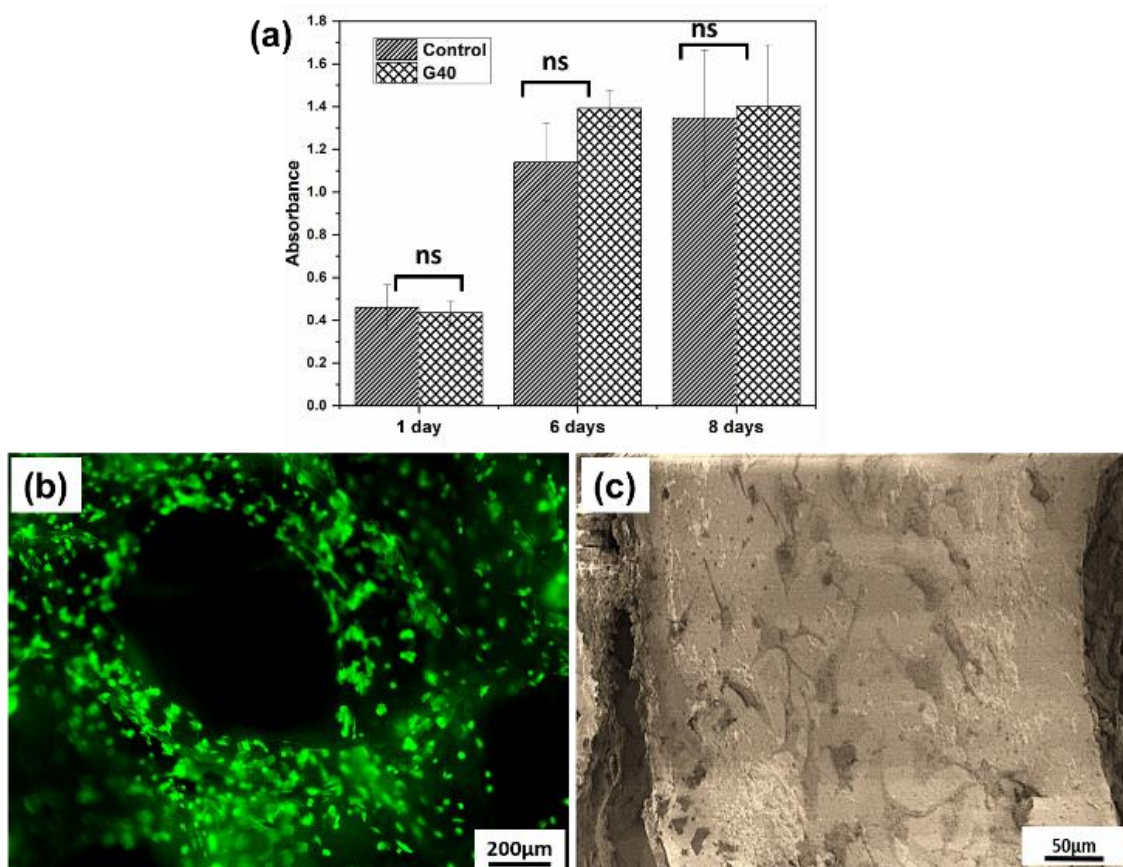


232 **Figure 4.** FTIR spectra (a) and swelling analysis (b) of the indicated 3D gelatin scaffolds (* $p < 0.05$).

233 As glutaraldehyde crosslinking might alter the cytocompatibility of gelatin scaffolds, the effect
234 of the scaffold on MG63 cell viability was analyzed with the MTT assay (Figure 5a). The viability of
235 MG63 cells grown in the presence of the G40 scaffold for up to 8 days was comparable to that of cells
236 cultured in the absence of scaffold (control). This proved that glutaraldehyde crosslinking did not
237 affect significantly cell viability.

238 Moreover, epifluorescence microscopy and SEM analysis after calcein staining showed that
239 MG63 cells could attach and proliferate on the G40 scaffold (Figure 5b and c). These findings confirm
240 the cytocompatibility of the G40 scaffold. In general, scaffolds are functionalized with cell adhesion
241 molecules to facilitate cell attachment. As gelatin contains the RGD (Arg-Gly-Asp) binding motif for

242 integrin [24, 25], no additional functionalization of these gelatin scaffolds is required for cell
243 attachment.



244

245 **Figure 5.** Cell viability and attachment on 3D gelatin scaffolds (G40) analyzed with the MTT assay (a),
246 and by calcein staining: epifluorescence microscopy image (b) and SEM image showing cells attached
247 on the G40 scaffold (c), ns=no significance (statistical analysis was performed using student t test).

248 4. Conclusions

249 The engineering of the scaffolds with an interconnected pore network and appropriate pore size
250 is essential to facilitate cell infiltration, and the transport of nutrients and wastes. Many studies
251 reported that scaffolds with a pore size higher than ~300 µm are optimal for bone tissue engineering
252 because they significantly reduce cell aggregation and facilitate cell proliferation and vascularization
253 [19, 26-29].

254 The work presented here shows that the cautious engineering of sacrificial molds allows the
255 fabrication of gelatin scaffolds with tunable pore size and architecture suitable for tissue engineering
256 applications. The synthesized gelatin scaffolds displayed interconnected pores with a pore size
257 between 400 and 1200 µm, and were stable in PBS as assessed by swelling assay. Assays using MG63
258 osteoblastic cells showed that such scaffolds are biocompatible, and allow cell attachment and
259 proliferation without addition of any cell adhesion molecule. In conclusion, this study provides a
260 method to develop engineered stable interconnected porous gelatin scaffolds using sacrificial mold-

261 assisted 3D printing. Further, this approach could be extended for the fabrication of customized
262 scaffolds using various other biopolymers or synthetic polymers. Human organs display multiple
263 biopolymeric composition as well as gradient composition in their scaffold matrix [30]. Synthesis of
264 such complex scaffolds could be attempted using sacrificial mold technique. Electrical simulation
265 found to improve the nerve tissue regeneration and conducting polymer-based particles are highly
266 biocompatible [31-34]. Such particles could be mixed with biopolymers for the conductive scaffolds
267 fabrication using sacrificial mold assisted technique. These scaffolds could be loaded with therapeutic
268 molecules and employed for wound healing and skin tissue engineering applications.

269

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276

277 **Conflicts of Interest:** The authors declare no conflict of interest.

278

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