

1    **Patient-Specific 3D Scanned and 3D Printed Antimicrobial Polycaprolactone Wound**  
2    **Dressings**

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18 **Abstract**  
19 The increasing prevalence of wound infections caused by antibiotic resistant bacteria is an urgent  
20 challenge facing modern medicine. To address this issue the expedient use of antimicrobial metals  
21 such as zinc, copper and silver were incorporated into an FDA-approved polymer (polycaprolactone -  
22 PCL) to produce filaments for 3D printing. These metals have broad-spectrum antimicrobial  
23 properties, and moreover, copper and zinc can enhance the wound healing process. 3D scanning  
24 was used to construct 3D models of a nose and ear to provide the opportunity to customize shape  
25 and size of a wound dressing to an individual patient. Hot melt extrusion was used to extrude pellets  
26 obtained by vacuum-drying of solutions of PCL and the different metals in order to manufacture  
27 metal-homogeneously-loaded filaments. Wound dressings with different shapes were produced with  
28 the filaments containing different concentrations of metals. Release of the metals from the dressings  
29 was determined by inductively coupled plasma atomic emission spectroscopy. All the different metal  
30 dressings show fast release (up to 24 h) followed by slow release (up to 72 h). The antibacterial  
31 efficacy of the wound dressings was tested using a thermal activity monitor system, revealing that  
32 silver and copper wound dressings had the most potent bactericidal properties. This study shows  
33 that 3D scanning and 3D printing, which are becoming simpler and more affordable, have the  
34 potential to offer solutions to produce personalised wound dressings.

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38 **Keywords:**  
39 3D printing  
40 3D scanning  
41 Wound dressings  
42 Polycaprolactone  
43 Additive manufacturing  
44 Personalised medicine  
45  
46

47 **1 Introduction**

48 The skin is the largest organ in the body, functioning as a sensory system, regulating both  
49 temperature and moisture transmission and acts as a physical barrier against the external  
50 environment. When a wound occurs, due to trauma or disease, the barrier becomes compromised.  
51 This can increase the susceptibility of the wound site to microbial infections originating from  
52 endogenous sources, such as surrounding skin and mucous membranes, or from exogenous sources,  
53 such as those introduced by injury or from the local environment (Landis, 2008). The introduced  
54 microorganism may overcome the host's defences and invade into deeper tissues, progressing to a  
55 more severe infection, thus causing further damage and delaying healing of the wound (Siddiqui and  
56 Bernstein, 2010).

57 A wound may require the application of an external dressing to temporarily compensate for the  
58 damaged barrier and to allow healing to initiate and progress. A wound dressing isolates the injury  
59 site from the external environment, and provides an optimal environment for the wound to heal by  
60 promoting haemostasis and limiting tissue oedema through external compression (Zahedi et al.,  
61 2010). Wound dressings, traditionally used to protect the wound from contamination, can be used  
62 as platforms to deliver actives to wound sites. The use of solid wound dressings is preferred to the  
63 use of topical bioactive agents in the form of solutions, creams, and ointments in the case of  
64 exudative wounds for drug delivery to the wound as they provide better exudate management and  
65 prolonged residence at the wound site. These dressings are potentially useful in the treatment of  
66 local infections being beneficial to achieve increased local concentrations of antibiotics while  
67 avoiding systemic treatment, thus reducing patient exposure to an excess of drug beyond that  
68 required at the wound site (Boateng and Catanzano, 2015).

69 Due to the alarming increase of multi-drug resistance bacteria worldwide, caused by the over-use  
70 and miss-use of antibiotics, the application of broad-spectrum antimicrobial agents such as metal  
71 ions is an attractive target. Having been used historically for their antimicrobial properties (Lemire et  
72 al., 2013; Tenaud et al., 2009), the use of inorganic antimicrobial metals in the fight against  
73 infections is of high importance due to the fact that they act on multiple bacterial pathways, which  
74 makes it difficult for the bacteria to develop resistance against them (Huh and Kwon, 2011). Silver is  
75 probably the most commonly used metal, but zinc and copper, two of the essential trace elements in  
76 the human body, are also known to play an integral part in the wound healing process.

77 Silver ions have been shown to bind to various bacterial cell membrane proteins to cause cell lysis,  
78 and can be transported into bacterial cells, where silver ions disrupt the cell wall to interfere with  
79 energy production, enzyme function, cell replication and ultimately cell death (Chopra, 2007; Fong  
80 and Wood, 2006; Jain et al., 2009). There remains a concern in relation to the toxicity of silver to  
81 humans, however, most frequent side effects including local skin irritation, discolouration or staining  
82 which are harmless and usually reversible (Cutting et al., 2007). Copper ions function by altering  
83 proteins and inhibiting their biological activity, membrane lipid peroxidation, and plasma membrane  
84 permeabilization (Borkow and Gabbay, 2005; Gabbay et al., 2006). Copper can improve the healing  
85 process as it plays a key role in the enhancement of angiogenesis, via induction of vascular  
86 endothelial growth factor (VEGF), up-regulating the activity of copper-dependent enzymes, cell  
87 proliferation and re-epithelialisation (Liu et al., 2009). It is suggested that the mode of action of ZnO is  
88 due to the disruption of bacterial cell membranes, and zinc is involved in several transcription  
89 factors and enzyme systems, stimulates the proliferation of epidermal cells, and increases collagen  
90 synthesis. Topical zinc can improve the healing of wounds especially in patients with zinc deficiency  
91 (Lemire et al., 2013), which can be a result of hereditary causes (Lansdown et al., 2007).

92 Wound dressings are usually prepared from absorbent, cross-linked polymer networks. One  
93 potential polymer is polycaprolactone (PCL), a semi-crystalline polyester that is biodegradable and  
94 biocompatible. These properties have led to the approval of several PCL drug-delivery devices and  
95 implants by the FDA (Salgado et al., 2012). It has a slow rate of degradation *in-vivo* compared with  
96 other biodegradable polyesters, a property that can be exploited in the manufacture of controlled  
97 release formulations (Li et al., 2014). PCL has been widely investigated in wound and burn dressings  
98 (Boateng et al., 2008; Ng et al., 2007), tissue engineering (Kweon et al., 2003), scaffold  
99 manufacturing (Kamath et al., 2014) and drug targeting (Freiberg and Zhu, 2004).

100 Three-dimensional printing (3DP) is a recently developed technology with numerous possibilities for  
101 the manufacture of medical devices. 3DP is an additive manufacturing process that allows the  
102 fabrication of three dimensional solid objects of virtually any shape. Of the several types of 3D  
103 printing, fused deposition modelling (FDM) has been most widely used for medical devices as it is  
104 simple, cost effective and extrudes polymer strands (Goyanes et al., 2016a; Yu et al., 2008). The  
105 printer feedstock is a thermoplastic filament that is heated to its softening point and then extruded  
106 through a print-head (driven by an X – Y orientation system) layer by layer over a build plate. The  
107 build plate is then lowered to a predetermined height and the process is repeated until the 3D  
108 object has been constructed. FDM 3DP has been used in various fields, such as tissue engineering,  
109 scaffold manufacturing (Fielding et al., 2012), and to produce oral drug delivery formulations  
110 (Goyanes et al., 2014; Goyanes et al., 2015a; Goyanes et al., 2016b; Goyanes et al., 2015b; Melocchi  
111 et al., 2015; Pietrzak et al., 2015). The ‘instructions’ for the 3D printer on how to build the object  
112 comes from the printer’s software that slices the source digital file into layers that form the  
113 instructions for the 3D printer. This digital file can be created using computer-aided design software,  
114 to construct a new 3D object, or with the use of 3D scanning, to copy an existing object. 3D scanning  
115 is a non-contact, non-destructive technology that digitally captures the shape of physical objects  
116 with a 3D scanner using laser light that collects distance information from surfaces. This information  
117 is then used to create ‘point clouds’ of data from the surface of the object. Hence, 3D laser scanning  
118 is a way to capture a physical object’s exact size and shape to construct a 3D model (Koch, 2012).  
119 The proof of concept of combining 3D printing and 3D scanning for the manufacture of antiacne  
120 masks/patches has been previously reported (Goyanes et al., 2016a), whereas the use of FDM  
121 printing showed high drug degradation due to the heating process while printing.

122 The combination of 3D printing and 3D scanning could possibly revolutionise patient care by  
123 allowing custom-manufacture of devices for individual patients and it is the exploration of this  
124 concept, applied specifically to wound dressings, that is the focus of this work. Hot melt extrusion  
125 was used to incorporate metal ions into a PCL filament and the 3D printer was used to fabricate  
126 dressings against scanned templates of a target wound. The antimicrobial efficacy of the dressings  
127 was also assessed using an *in-vitro* assay.

128

## 129 **2 Materials and Methods**

### 130 **2.1 Materials**

131 PCL pellets ( $(C_6H_{10}O_2)_n$ , Mw ~ 80,000) and silver nitrate ( $AgNO_3$ ) were purchased from Sigma-  
132 Aldrich, UK. Copper sulphate (II) pentahydrate ( $CuSO_4 \cdot 5H_2O$ ) was purchased from VWR chemicals,  
133 Belgium. Zinc oxide ( $ZnO$ ) was purchased from Alfa Aesar, USA. The test organism *Staphylococcus*  
134 *aureus* (NCIMB 9518) was purchased from Fisher Scientific, UK. Nutrient broth (CM0001) was  
135 purchased from Thermo Scientific, UK.

136

137 **2.2 Methods**

138 **2.2.1 Preparation of metal loaded filaments**

139 - Silver-loaded filament (10% loading w/w):

140  $\text{AgNO}_3$  (3 g) was dissolved in 10 mL of deionized water using a magnetic stirrer. Tetrahydrofuran  
141 (THF, 200 mL) was added to the silver solution under stirring. Finally, 27g of PCL pellets was then  
142 added to the solution and the mixture was stirred at 40 °C until complete dissolution of PCL. The  
143 solvents were removed with a rotary evaporator under reduced pressure at 40 °C for 2 h followed by  
144 high-vacuum drying for 1h. The dried material ( $\text{AgNO}_3$  homogeneously distributed in the PCL) was  
145 chopped into pellets and extruded with Filabot filament hot-melt extruder (Filabot Inc, USA) with a  
146 single screw and a 1.75 mm nozzle head. The extrusion temperature was 80 °C.

147

148 - Copper-loaded filament (10 and 25% loading w/w):

149  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (3g or 7.5g for 10% or 25% loading respectively) was dissolved in 100mL methanol using  
150 a magnetic stirrer. PCL pellets (27 g or 22.5 g for 10% or 25% copper loading respectively) was then  
151 added to the copper solution, followed by 100mL dichloromethane (DCM) and the mixture was  
152 stirred at 40°C until complete dissolution of PCL. A rotary evaporator (under reduced pressure) was  
153 used to evaporate the solvents at 40 °C for 3 h followed by high-vacuum drying for 1 h. The dried  
154 material ( $\text{CuSO}_4$  homogeneously distributed in the PCL) was chopped into pellets and extruded with  
155 Filabot filament hot-melt extruder (Filabot Inc, USA) with a single screw and a 1.75mm nozzle head.  
156 The extrusion temperature was 60°C.

157

158 - Zinc-loaded filament (10 and 25% loading w/w):

159  $\text{ZnO}$  (3g or 7.5g for 10% or 25% zinc loading respectively) was dissolved in 100 mL ethanol using a  
160 magnetic stirrer. PCL pellets (27g or 22.5g for 10% or 25% copper loading respectively) was added  
161 followed by 100 mL DCM and the mixture was stirred at 40°C until complete dissolution of PCL. The  
162 solvents were removed using a rotary evaporator at 40 °C for 3 h followed by one hour high-vacuum  
163 drying. The dried material ( $\text{ZnO}$  homogeneously distributed in the PCL) was chopped into pellets and  
164 extruded with Filabot filament hot-melt extruder (Filabot Inc, USA) with a single screw and a  
165 1.75mm nozzle head. The extrusion temperature was 75°C.

166

167 For all the filaments prepared the diameter of the filament was checked using a digital calliper  
168 throughout the extrusion process, since it is important to get a consistent filament diameter within  
169 an acceptable range for the 3D printer.

170

171 **2.2.2 3D Scanning**

172 3D scans were captured with a Sense 3D Scanner (3D Systems, USA). It functions by capture of the  
173 surface data of a physical object reflected light from a laser. In this work, scans were captured of a  
174 nose and ear, because 3D printed dressings of these body parts can dress anatomically complex  
175 areas compared to conventional flat dressing, what would provide more comfort to the patient. The  
176 settings used were high resolution, with object recognition enabled, colour scanning and landscape  
177 orientation. The person being scanned was in a setting position, while the person holding the 3D  
178 scanner was rotating 360°around the subject while maintaining about 40 cm distance to the  
179 subbjetct. These 3D scans were cut, optimized for 3D printing and templates were made using  
180 Autodesk Meshmixer 10.8.

181 **2.2.3 3D Printing**

182 A MakerBot Replicator 2X Desktop 3D printer (MakerBot Inc., USA) was used to print wound  
183 dressings shaped to match the nose and ear scans, in addition to square dressings (20 x 20 x 1 mm)  
184 for antimicrobial studies and circular dressings (10 mm diameter x 1 mm thickness) for dissolution  
185 testing. The templates for the square and circular dressings were created using Tinkercad (Autodesk)  
186 – a browser-based 3D design and modelling tool.

187  
188 The nozzle head was cleaned (for 20 – 25 s) prior to printing the metal-loaded filaments, and  
189 between prints containing different metal ions or different concentrations, by extruding plain PCL  
190 filament. The settings of the printer, which will ultimately determine how the 3DP dressings will turn  
191 out, were selected based on preliminary results with the metal loaded filaments. All the dressings  
192 were printed at an extrusion temperature of 170 °C, high resolution (0.1 mm layer height), with two  
193 shells, 100% infill and speed while extruding and while travelling was set to 50 mm/s. A raft and  
194 support were used for the printing of the nose and ear dressings, while no support or raft was used  
195 for the printing of the flat dressings printed for analytical purposes.

196  
197 **2.2.4 Thermal characterisation of metal-loaded filaments and dressings**

198 Differential scanning calorimetry (DSC): Measurements of the metal loaded filaments and the 3D  
199 printed dressings were performed using a TA Q2000 DSC (TA Instruments LLC, USA), calibrated with  
200 indium ( $T_m = 156.6$  °C,  $\Delta H_f = 28.71$  J/g). Nitrogen gas was used as a purge with a flow rate of 50  
201 mL/min. Tzero hermetic pans with lids were used for all samples, with an average sample weight of  
202 7.9 mg. Samples were cooled to -80 °C then heated to 200 °C at a heating rate of 10 °C/min.

203  
204 Thermogravimetric analysis (TGA): TGA analysis was performed with TA Discovery TGA (TA  
205 Instruments LLC, USA) with nitrogen as purge (flow rate = 25 mL/min). Open aluminium pans were  
206 used, and samples were heated from room temperature ( $15 \pm 0.5$  °C) to 200 °C at 10 °C/min.

207  
208 **2.2.5 Scanning electron microscopy (SEM)**

209 Surface and cross-section images of the filaments were taken using JSM-840A Scanning Microscope,  
210 JEOL GmbH, Germany. The voltage and working distance were set at 5 kV and 50 mm, respectively.  
211 Filament samples were placed on double-sided carbon tape, mounted on stubs and sputter coated  
212 using a Polaron E5000 machine with Au/Pd. Samples were coated for 1 minute prior to imaging.

213  
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216 **2.2.6 Fourier transform infrared (FTIR) spectroscopy**

217 FTIR spectra of Ag, Cu and Zn powder, filaments and dressings were acquired using Bruker ALPHA  
218 Platinum FT-IR spectrometer (USA) to determine if Ag, Cu or Zn form any bonding with the  
219 polycaprolactone matrix. Spectra were acquired at  $4000\text{ cm}^{-1}$  to  $400\text{ cm}^{-1}$  and a resolution of  $2\text{ cm}^{-1}$ .

220  
221 **2.2.7 Dissolution testing of wound dressings**

222 For each assay the dressing was placed into a sterile 10 mL vial with agitation in the dissolution  
223 medium (10 mL of 0.1 M phosphate buffer – pH 7.4). The vials were capped and incubated in a  
224 thermostated bath at 37 °C for three days. At regular intervals (0, 6, 12, 18, 24, 36, 48, 60 and 72 h),  
225 1 mL aliquots were sampled from each vial and replaced with an equal amount of phosphate buffer.  
226 The samples were then diluted to 5 mL with 96% (w/w) nitric acid (to digest any dissolved polymer  
227 matrix), stirred at room temperature for 1 h, then 1 mL was taken from that solution and diluted  
228 further to 20 mL with phosphate buffer.

229 Analysis of the samples was performed with Inductively Coupled Plasma Atomic Emission  
230 Spectroscopy (ICP-AES) using an Axial Varian 720-ES, with argon as a purge gas. Ag was analysed at  
231 wavelength 328.068 nm, Cu at 327.395 nm and Zn at 213.857 nm. A second wavelength (338.289 nm  
232 for Ag, 324.754 nm for Cu and 202.548 nm for Zn) was used to confirm the reproducibility of the  
233 results. Each dressing was tested in triplicate and the mean value determined.

234

### 235 **2.2.8 Antibacterial efficacy of wound dressings**

236 Antibacterial efficacy of wound dressings was tested against *S. aureus* which is a common bacterium  
237 to causes skin infections. *S. aureus*, stored in 1 mL aliquots at -80 °C in 15% w/v glycerol, was  
238 defrosted at 37 °C and used to inoculate nutrient broth, which was incubated overnight aerobically  
239 at 37 °C. Bacteria were harvested from the broth by centrifugation at 3000 g for 10 min and washed  
240 in phosphate buffered saline (PBS) (Fisher Scientific, UK) three times. The resulting bacterial  
241 suspension was adjusted to a 0.5 McFarland standard using PBS to standardize the cell numbers to  
242 approximately 1 x 10<sup>8</sup> cfu/mL. This was verified with serial dilution and spread plating.

243

244 Dressing samples (plain PCL, Ag-PCL, Cu-PCL, and Zn-PCL) printed with identical settings (see 3D  
245 printing above) were cut to the required weights (10, 20, 25, 30, 40, 50, 75 and 100 mg) immediately  
246 prior to use and inserted into a sterile 3 ml calorimetric ampoule (Hichrom, UK). Nutrient broth  
247 (2.97 mL) was added to the ampoule, followed by inoculation with the bacterial suspension (30 µL).  
248 The ampoule was then sealed with a crimp cap. A control ampoule was prepared for each  
249 experiment containing only nutrient broth and the same inoculum of bacteria. The ampoule was  
250 vortexed briefly before being transferred to a 2277 Thermal Activity Monitor (TAM, TA Instruments  
251 Ltd, UK).

252

253 The ampoules were allowed to equilibrate for up to 30 min before being lowered into the measuring  
254 position of the TAM (set at 37 °C with an amplifier setting of 1000 µW). Digitam 4.1 software  
255 collected heat output data every 10 s for 48 h.

256

257 After calorimetric analysis, ampoules were removed from the TAM and inspected for turbidity. Non-  
258 turbid ampoules were vortexed for 10 s then opened and 1 mL of nutrient broth removed to  
259 enumerate the bacteria. The sample was centrifuged at 3000 g for 10 min and resuspended in PBS  
260 three times in an attempt to remove any metal ions that could affect the growth of the bacteria on  
261 agar. The resulting bacterial suspension underwent serial dilution and spread plating on ISA,  
262 followed by incubation overnight at 37 °C. Colonies were counted, and the number of viable bacteria  
263 in the ampoule calculated.

264

### 265 **3 Results and Discussion**

266 The manufacture of metal-loaded filaments for 3D printing was achieved with PCL. Five metal loaded  
267 PCL filaments were produced with different concentrations: Ag (10% w/w)-PCL, Zn (10% w/w)-PCL,  
268 Zn (25% w/w)-PCL, Cu (10% w/w)-PCL and Cu (25% w/w)-PCL (Figure 1). The average filament  
269 diameter was 1.77 ± 0.3mm. The metal compounds and the PCL were dissolved in an appropriate  
270 solvent mixture and the solution vacuum-dried to obtain pellets with a homogeneously distribution  
271 of the metal compound into the PCL.

272

273 One of the most challenging parts of the process was the determination of a common solvent to  
274 dissolve both the metal and polymer. For instance,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{AgNO}_3$  were soluble in water  
275 while  $\text{ZnO}$  and PCL were insoluble in water. It was established that a single solvent was not adequate  
276 to dissolve both the polymer and any of the metals so ultimately a combination of solvents was used  
277 to dissolve PCL and the metals (see Methods for the exact combination for each preparation). This  
278 method is cheap, versatile and only requires the selection of suitable solvents, moreover the direct  
279 extrusion of the metal compounds and PCL is not recommended since that lead to filaments with a  
280 very poor distribution of the metal compounds in the PCL.

281 Two factors were critical in ensuring extrusion produced a filament of consistent diameter. Firstly,  
282 the extrusion temperature varied depending on the mixture content and how dry the mixture was.  
283 Copper-containing mixtures required a lower extrusion temperature ( $60\text{ }^\circ\text{C}$ ) compared with the non-  
284 copper-containing mixtures (which were extruded at  $75 - 80\text{ }^\circ\text{C}$ ). This could be due to the lower  
285 melting temperature of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  ( $110\text{ }^\circ\text{C}$ ) compared with  $\text{AgNO}_3$  ( $212\text{ }^\circ\text{C}$ ) and  $\text{ZnO}$  ( $1975\text{ }^\circ\text{C}$ ).  
286 When the extrusion temperature was lower than required, it led to a thicker filament and/or  
287 clogging of the extruder. Higher extrusion temperatures led to the extrusion of filaments that were  
288 thin and inconsistent (about  $1.35 \pm 0.05\text{ mm}$ ). Secondly, a regular feeding rate was required to  
289 produce a uniform filament diameter.

290 SEM micrographs of the filaments revealed that all the filaments had homogenous and uniform  
291 surface and cross section, indicating uniform metal distribution inside the filaments (Figure 2).

292 3D templates for wound dressings were successfully obtained from 3D scanning. The resolution of  
293 the 3D scanner was one of the main factors determining the quality of the 3D scans, however,  
294 lighting conditions (e.g. direct sunlight) and room temperature did affect the depth of acquisition of  
295 the scanner.

296 Ag, Cu and Zn loaded PCL dressings were printed. Figure 3 shows an example of a Cu-PCL printed  
297 nose dressing (see Appendix 1 for more examples). All the dressings were flexible, most likely due to  
298 the elastomeric properties of PCL. These 3D printed dressings have an advantage over conventional  
299 flat dressing in that they can dress anatomically complex areas. This would provide more comfort to  
300 the patient and improve adherence. The cytocompatibility of PCL in addition to the possibility of  
301 incorporating bioactive or antimicrobial agents means that PCL has the potential to be tailored into  
302 an effective wound dressing with appropriate bio-physical properties (e.g. vapour permeability and  
303 flexibility) and personalised shape and size. The use of metal ions improves the printing performance  
304 of the PCL filaments. In a previous study using PCL filaments loaded with salicylic acid for the  
305 treatment of acne, the 3D printer was able to manufacture flat disc/patches but not complex shapes  
306 as personal shape devices (Goyanes et al., 2016a).

307 FTIR spectra of plain PCL pellets (before 3D printing) exhibited both absorption bands of the  $-\text{C}-\text{H}$   
308 and  $\text{C}=\text{O}$  functional groups at  $2942\text{ cm}^{-1}$  and  $1722\text{ cm}^{-1}$  respectively (Figure 4). FTIR analysis of the  
309 printed plain PCL, Ag(10%)-PCL, Cu(10%)-PCL, Cu(25%)-PCL, Zn(10%)-PCL and Zn (10%)-PCL showed  
310 all absorption bands of the functional groups ( $-\text{C}-\text{H}$  and  $\text{C}=\text{O}$ ). In addition, there was no shift in peak  
311 positions of the metal-loaded 3D printed samples compared to plain PCL pellets or 3D printed PCL,  
312 indicating that there was no chemical bonding between PCL, Ag, Cu or Zn had occurred during  
313 extrusion and printing of the dressings.

314 The main limiting factor in printing good dressings was a consistent filament diameter within an  
315 acceptable range for the 3D printer. A filament thin in sections resulted in areas of the dressings  
316 containing less material than other areas, and thicker filament sections were too difficult for the

317 extruder head to grip. Thus, after various experimentations with slight variations in filament  
318 diameters, it was determined that the consistency of the filament diameter (1.69 – 1.77 mm) was  
319 more important than the size of the diameter (given that it is within acceptable range of the 3D  
320 printer; 1.60 – 1.79 mm).

321 3D printing of wound dressings of good quality requires an understanding of the settings that will  
322 ultimately dictate how they would turn out. For dressings printed in this work, these settings were  
323 the layer height, number of outer shells and both speed while extruding and travelling. These  
324 settings, individually or combined, did directly control the surface finish, density and quality of the  
325 final print.

326 Increasing the number of shells (the outer most layers of the print) provided stronger dressings;  
327 however, they increased printing time and reduced quality (e.g. 4 shells resulted in substantial  
328 reduction of details and inconsistent surface of printed dressings compared to 2 shells). Having too  
329 few shells resulted in a weak and fragile print. There needs to be a balance between not having  
330 enough or too many shells, and in this case, the default of two outer shells was a good compromise.

331 The resolution of the 3D printed dressing is determined by the layer height. Using a smaller layer  
332 height provided a considerable increase in detail and increased printing time. The MakerBot  
333 Replicator 2X can print in layer heights between 0.1 mm and 0.3 mm, however it was only possible  
334 to obtain good quality prints with 0.1 mm layer heights. One reason for this is because 3D prints  
335 made with FDM printers typically have visible ridges between different layers, and a smaller layer  
336 height helps to reduce (but not eliminate) them.

337 The printing temperature in addition to both printing and movement speed determine if it is  
338 possible to print at all. The extrusion temperature depends on the filament material being used, for  
339 instance, plain PCL dressings could be printed with as low as 140 °C. However, when PCL is loaded  
340 with metals, it was not possible to print until this temperature was increased to 170 °C. High  
341 movement speed while printing or travelling reduced the printing time by making the print-head  
342 move faster, but resulted in poorer print quality. On the other hand, slower speed meant that the  
343 hot print-head would stay longer above the extruded layers resulting in burnt layers, especially the  
344 last layers. The optimal settings found in this case was 50 mm/s for both printing and travelling  
345 speeds.

346  
347 The DSC thermogram (Figure 5) shows that plain PCL dressing has a melting temperature ( $T_m$ ) of  
348 60.9°C and a glass transition temperature ( $T_g$ ) of -63.4 °C which agrees with the literature values of  
349 PCL pellets (60.0 °C and -60.0 °C respectively) (Hutmacher et al., 2001). All the metal loaded  
350 dressings show similar thermal profiles compared with the plain PCL dressing without any  
351 degradation at temperatures up to 200 °C. Ag-PCL had the lowest  $T_m$  (59.4 °C) while Zn-PCL had the  
352 highest (61.8 °C). Ag-PCL dressing decreased the  $T_m$  while both Zn-PCL and Cu-PCL dressings  
353 increased it slightly, these changes did not have effect on the printability of the filaments.

354 TGA showed no significant mass loss (0.44% to 1.89%), most likely due to loss of residual solvents.  
355 Copper-containing dressings (10 and 25% w/w) showed the highest amount of weight loss (1.63%  
356 and 1.89% respectively) compared with the other dressings. This could be due to the hygroscopicity  
357 of copper sulphate. This might become an issue in the future during storage and transport of the  
358 dressings. However, with proper storage conditions and packing this concern can be overcome.

359 Thus it can be concluded that the thermal analysis results confirm that the printed dressings were  
360 stable and that the printing and extrusion processes did not affect the properties of PCL. It is

361 important to note that even though the residence time of the formulation in the print head is short  
362 (a few seconds), thermally labile formulations may experience some degree of degradation during  
363 the printing process (Goyanes et al., 2016a). Hence, DSC analysis may be used to assess the  
364 suitability of the formulation for FDM 3D printing (Goyanes et al., 2015a).

365 One of the challenges in antimicrobial research for wound dressings is achieving sustained release of  
366 the antimicrobial agent for extended prevention of bacterial infection. The release of Ag, Cu and Zn  
367 from PCL dressings to the surrounding environment is shown in Figure 6. During the first 24 h of the  
368 experiment, Ag was released very quickly (40.69 µg/mL), but the release rate decreased rapidly in  
369 the following 24 h reaching a concentration of 44.53 µg/mL at 48h. From 48 to 72 h the  
370 concentration of Ag remained almost constant at 45.85 ± 1.10 µg/mL. The fast release observed in  
371 the first 24 h is most likely the release of Ag from the surface of the PCL matrix, and the slower  
372 release afterwards is due to the slow diffusion of Ag from the interior of the polymer matrix to the  
373 surface before release. The final concentration (44.53 µg/mL) is two folds higher than the minimum  
374 inhibitory concentration and minimum bactericidal concentration previously reported for silver  
375 against *S. aureus* (22.083 µg/mL) (Said et al. 2014).

376

377 Over the same time period (0 – 72 h) the concentration of Cu and Zn had the same trend but was  
378 always much lower compared to Ag. The release rate was highest for 10% Ag-PCL (45.85 µg/mL) and  
379 lowest for 10% Zn-PCL (15.87 µg/mL). Both 25% Cu-PCL and 25% Zn-PCL had higher release rate  
380 compared to their corresponding 10% dressings. This is due to the fact that the metal content in the  
381 25% dressing is higher than the 10% leading to more metal being released. 25% Cu-PCL had higher  
382 release rate than 25% Zn-PCL (same applies for the 10% dressings of both metals). Minimum  
383 inhibitory concentration for copper against *S. aureus* was reported to be between -3 – 40 µg/ml,  
384 being the minimum bactericidal concentration between 7 and 60 µg/ml (Argueta-Figueroa et al.  
385 2014). The amount of copper released from the dressings was 17.756 µg/ml (for 10%) and 26.634  
386 (for 25%), values which fall in the middle of the reported values. However, the antibacterial efficacy  
387 of Zn and Cu is dependent on the concentration of the metal, the initial bacterial concentration, and  
388 the strains of bacteria employed in the study.

389 The minimum inhibitory concentrations found in the literature for Zn against *S. aureus* are very  
390 variable and not comparable to the test performed in this study. Zn nanoparticles vs *S. aureus*  
391 showed a minimum inhibitory concentration determined by agar dilution method of 625 µg/ml  
392 (Aleaghil et al. 2016). The highest concentrations obtained were 15.87 µg/ml for 10% and 20.63  
393 µg/ml for 25% Zn wound dressings, which are significantly lower than the concentration reported.  
394

395 The controlled release of Ag, Cu and Zn from PCL dressings is attributed to the entrapment of the  
396 metals into PCL, which acts as a barrier for the release of these metals from the dressing due to the  
397 slow water penetration into the PCL matrix. These results confirm that entrapment of metal ions  
398 into PCL dressings delays the release of the metals. This is desirable to maintain sufficient release of  
399 antimicrobial agent to remain active for the duration of treatment, while preventing high  
400 concentrations to be released upon initial application which would prevent adverse events (such as  
401 irritation) from high doses. Another advantage of a slower and prolonged release rate of Ag, Zn and  
402 Cu in clinical practice is that it would reduce the number of dressing changes, which can be very  
403 painful (Meaume et al., 2004).

404

405 These results are in agreement with the solubility of the metals in water (majority constituent of the  
406 phosphate buffer testing medium) where Ag has superior solubility properties, followed by Cu then  
407 Zn. Ideally, the dissolution testing could have been performed in the same medium used in the

408 antibacterial testing. This could give a better correlation between release profiles of the metals from  
409 dressings and antibacterial activity. However, that was not possible as the nutrient broth used for  
410 antibacterial testing contains NaCl which led to the precipitation of solid AgCl when Ag-PCL dressing  
411 was dissolved in the medium during the initial experiments. Moreover, pH 7.4 of the phosphate  
412 buffer resembles the pH at surface of the skin providing closer correlation to the *in-vivo*  
413 environment.

414 Isothermal micro-calorimetry (IMC) was used to quantitatively monitor the efficacy of silver, zinc and  
415 copper in wound dressings. IMC monitors the rate of heat production (power) in a sample, where  
416 the power signal is proportional to the number of viable cells in the sample. This allows for real-time  
417 measurement of the growth (or inhibition) of *S. aureus*, without being affected by non-viable cells.  
418 This method is not dependent on optical clarity (which can be effected by the presence of the metal  
419 ions in the sample), and does not require the organism to be removed from its environment to be  
420 sampled (Gaisford et al., 2009; O'Neill et al., 2003). The drawback of IMC is that because heat is  
421 absorbed or produced by different events occurring in the sample, could mean that the power signal  
422 measured is potentially a combination of several processes. However, a careful experimental design  
423 can improve these issues as discussed by S. Gaisford et al. (Gaisford, 2005).

424 The control experiments of *S. aureus* (without any dressing or metals) shows a characteristically  
425 complex pattern, exhibiting an exponential growth phase in the first few hours with two distinctive  
426 biphasic peaks, during which heat is generated and an increase in power is recorded (Figure 7A). The  
427 area under the curve (AUC – total heat output) of the controls is reproducible ( $n = 3$ ) to 3.5%. As  
428 discussed by Zaharia et al. (2013), the first exponential phase (0 – 3 h) represents aerobic  
429 metabolism where the available oxygen (blue arrow in Figure 7A), dissolved in the medium is utilised  
430 (the ampoules are sealed but not completely filled to the top). This is then followed by a change in  
431 aerobic metabolism (3 – 10 h) using diffused oxygen from the head space of the ampoule (red arrow  
432 in Figure 7A). The last peak of the thermogram represents anaerobic metabolism of the organism  
433 using any remaining carbon sources that the organism is able to metabolise (green arrow in Figure  
434 7A) (Zaharia et al., 2013). The exhaustion of nutrients, pH drift and the appearance of toxic  
435 metabolites consequently stopping the organism from growing anymore. This resulted in the power  
436 signal to return to baseline (zero) and hence decided the 48 hour duration of the experiment.

437  
438 In the presence of plain 3D PCL dressings (i.e. the dressings with no antimicrobial metal agent), PCL  
439 showed no effect on the initial aerobic phase and the overall growth is very similar to that of the  
440 control (Figure 7B). There was very slight variation in the second growth phase which was attributed  
441 to microorganism cells becoming entrapped within the PCL dressing. Therefore, diffusion of medium  
442 to those trapped cells and metabolites from those microorganisms to the medium will be different  
443 compared to those present in the surrounding medium only. Thus, it can be concluded that PCL does  
444 not have any intrinsic antimicrobial properties, and increasing amounts of PCL does not affect the  
445 growth of *S. aureus*.

446  
447 The shape of the growth curve is significantly different in the presence of 10% (w/w) Ag-PCL dressing  
448 (Figure 7C). Use of 10 mg of Ag-PCL dressing delayed the growth by *ca.* 16 h, and inhibition of growth  
449 was observed when larger masses (20, 30 and 40 mg) were used when compared to the control.  
450 Viable counts at the end of each experiment (Table 1) confirmed a bactericidal effect on the  
451 bacteria, with a three log reduction in bacteria compared to the inoculum. These results indicate  
452 that silver dressing is effective at inhibiting the growth of *S. aureus* via a bactericidal mechanism, and  
453 that increasing amount of silver causes a more potent inhibition.

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Table 1. Viable cell counts after the IMC study

Formulation		10% Ag - PCL			
Mass of the dressing	Control (0g)	10 mg	20 mg	30 mg	40 mg
Viable cells	140,333	105	20	0	0
Formulation		10% Cu - PCL			
Mass of the dressing	Control (0g)	25 mg	50 mg	75 mg	100 mg
Viable cells	121,667	120,311	95,038	56,664	51,682
Formulation		25% Cu - PCL			
Mass of the dressing	Control (0g)	25 mg	50 mg	75 mg	100 mg
Viable cells	120,660	14,738	1,202	220	48
Formulation		10% Zn - PCL			
Mass of the dressing	Control (0g)	25 mg	50 mg	75 mg	100 mg
Viable cells	119,333	117,855	117,439	111,538	89,795
Formulation		25% Zn - PCL			
Mass of the dressing	Control (0g)	10 mg	20 mg	30 mg	40 mg
Viable cells	119,916	84,764	81,297	71,859	68,315

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469 Figure 7D shows the corresponding growth curves for *S. aureus* in the presence of increasing masses  
470 of 10% (w/w) Cu-PCL. All the samples (10 – 40 mg) showed no inhibition of the microorganism. This  
471 indicates that at this concentration Cu is ineffective at inhibiting the growth of *S. aureus* after 48 h,  
472 due to a slow release rate or the concentration of Cu is not sufficient. Hence, 25% (w/w) Cu-PCL was  
473 tested to determine if there is any improvement with higher concentrations of Cu (Figure 7E).  
474 Several differences from the control are apparent, but the interpretation of these data is difficult.  
475 There is an absence of any of the characteristic growth peaks of *S. aureus*, with high-power peaks at  
476 the beginning with an immediate sharp decline in power instead. There was no growth in any of the  
477 samples, which was confirmed by the non-turbidity of all the samples after the TAM experiments. In  
478 addition, viable counts revealed that a 25 mg dressing showed a two log reduction in viable bacteria  
479 (while higher masses of the dressing had stronger inhibition) at the end of the TAM experiment  
480 compared to the initial inoculum (Table 1). This suggests that Cu is effective at inhibiting the growth  
481 of *S. aureus*, although higher concentrations are required compared to silver. In efforts to explain

482 the unusually high peaks at the start of the growth curves are due to Cu, the bacteria or an  
483 interaction between any of dressings' content's and that of the medium, copper sulphate powder  
484 only (without any bacteria or PCL) was tested exactly as the Cu-PCL dressing in water and broth. As  
485 can be seen in Figure 7F, both curves show a similar pattern to that of the 25% (w/w) Cu-PCL. This  
486 confirms that these peaks are due to copper sulphate powder and are not due to any interaction  
487 between the dressing content, bacteria or the medium.

488  
489 Both 25 and 50 mg of 10% (w/w) Zn-PCL dressing showed no effect on the growth of *S. aureus* after  
490 72 h (Figure 7G). While 75 mg and 100 mg of the dressing showed a small reduction in the intensity  
491 of the growth peaks, and a minor delay of the growth. These results suggest that at these  
492 concentrations Zn is ineffective at inhibiting the growth of *S. aureus*, as confirmed by cell counting  
493 (Table 1).

494 The results with 25% (w/w) Zn-PCL show stronger inhibition compared to the 10% (w/w) Zn-PCL  
495 dressing (Figure 7H). Both 10 and 20 mg of 25% Zn-PCL showed similar inhibition, which was weaker  
496 compared to 30 and 40 mg of the dressing. In addition, there was a time delay of the growth (34 –  
497 82 min). These results suggest that increasing the concentration of Zn to 25% (w/w) increases the  
498 inhibition, however, it is not as effective as Ag or Cu. This is most likely due to a weaker bactericidal  
499 efficacy and lower release rate of Zn compared to Ag and Cu. Consequently, higher amounts of Zn  
500 may be required to be incorporated into the dressing to compensate for the low release rate and  
501 efficacy to achieve similar inhibition of Cu or Ag. This may present certain difficulties during  
502 formulation and an increase in cost. However, this may not be required as Zn can be incorporated  
503 into the dressing to benefit from its healing properties (especially in patients with zinc deficiency), in  
504 addition to the weaker antimicrobial efficacy.

505 It is important to make some clarifications regarding the nature of the assay method (IMC) used in  
506 this work. Any *in vitro* method will differ from the *in vivo* event, and the relevancy of these  
507 differences will depend on how the data is used. In this case, the *in vivo* environment is extremely  
508 difficult to reproduce. In a wound environment, bacteria can grow as biofilms or micro-colonies  
509 rather than planktonic cultures which can influence the susceptibility of the microorganism to an  
510 antibacterial agent (James et al., 2008). For instance, it has been suggested that the bactericidal  
511 concentration of silver required to eradicate biofilms of *Pseudomonas aeruginosa* is 10 to 100 fold  
512 higher than what is required to eradicate planktonic bacteria (Bjarnsholt et al., 2007). This would  
513 suggest that the concentrations used in this work might need to be increased to eradicate biofilms,  
514 since in the experiments reported here, the organism is growing in planktonic culture. In addition,  
515 the antimicrobial effect of metal ions is known to be strain dependent (Ruparelia et al., 2008). It is  
516 important to note that the metal release would be lower in skin versus suspending solution,  
517 although the release could be promoted increasing the metal loading in the filaments, so in the 3D  
518 printed wound dressings as shown in the ICP data. It is already reported that increasing the drug  
519 loading in 3D printed formulations increased drug release since there is less matrix compound (in  
520 this case PCL) avoiding the release of the active compounds (Goyanes et al., 2016b). The main aim of  
521 the microbiology experiments was to evaluate the efficacy of the metal loaded PCL wound dressings  
522 against a known skin pathogen (*S. aureus*), and to gain insights on how 3D printing might influence  
523 the outcome.

524 Since optimal moisture content maintains the vitality of tissue and promotes wound healing,  
525 theoretically, it would be possible to modify the thickness of the wounds dressings or to create  
526 regions with small gaps between the layers to modify the vapour permeability.

527

528 **4 Conclusion**

529 The results clearly demonstrate the utility of hot melt extrusion as a novel method to incorporate  
530 antimicrobial Ag, Cu and Zn into polycaprolactone filaments that allow the 3D printing of  
531 personalised wound dressings. 3D printed dressings demonstrated a clear advantage over  
532 conventional flat dressings as they are anatomically adaptable. This method takes advantage of 3D  
533 scanning to create 3D models of body parts which are then 3D printed in a personalised therapy. Ag-  
534 PCL and Cu-PCL dressings showed the most bactericidal properties against *S. aureus* which is a  
535 common bacterium to causes skin infections. This study therefore demonstrates a simple method to  
536 produce customizable wound dressings that can be tailored to individual patients in regards to  
537 shape, size and antimicrobial agents.

538

539 **5 Acknowledgment**

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541 assistance with the ICP-AES machine.

542

543 **6 References**

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639 **Figure Captions**

640 **Figure 1.** Filaments loaded with metals produced, from left to right: plain PCL, Ag (10% w/w)-PCL, Zn  
641 (10% w/w)-PCL, Zn (25% w/w)-PCL, Cu (10% w/w)-PCL and Cu (25% w/w)-PCL.

642 **Figure 2.** SEM images of: (A) plain PCL, (B) Ag (10% w/w)-PCL, (C) Cu (10% w/w)-PCL, (D) Cu (25%  
643 w/w)-PCL, (E) Zn (10% w/w)-PCL and (F) Zn (25% w/w)-PCL.

644 **Figure 3.** 3D scan model of a nose (left) and the printed wound dressing of this model with Cu-PCL  
645 (right).

646 **Figure 4.** FTIR spectra of the 3D printed dressings.

647 **Figure 5.** DSC analysis of indicated PCL wound dressings. Exothermic up.

648 **Figure 6.** Dissolution profiles of Ag (10% w/w)-PCL, Cu (10% w/w)-PCL, Cu (25% w/w)-PCL, Zn (10%  
649 w/w)-PCL and Zn (25% w/w)-PCL in phosphate buffer (pH 7.4).

650 **Figure 7.** Growth of *S. aureus* by showing the power generated of bacterial cells vs. time in the  
651 presence of increasing amount of dressing containing: (A) control experiments with no PCL or any  
652 metal ions, (B) plain PCL, (C) Ag (10% w/w)-PCL, (D) Cu (10% w/w)-PCL, (E) Cu (25% w/w)-PCL, (F)  
653 control experiment of plain CuSO<sub>4</sub> powder in broth and water without any PCL or bacteria, (G) Zn  
654 (10% w/w)-PCL and (H) Zn (25% w/w)-PCL. All experiments were performed at 37 °C over 48 h.

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657 Figure 1

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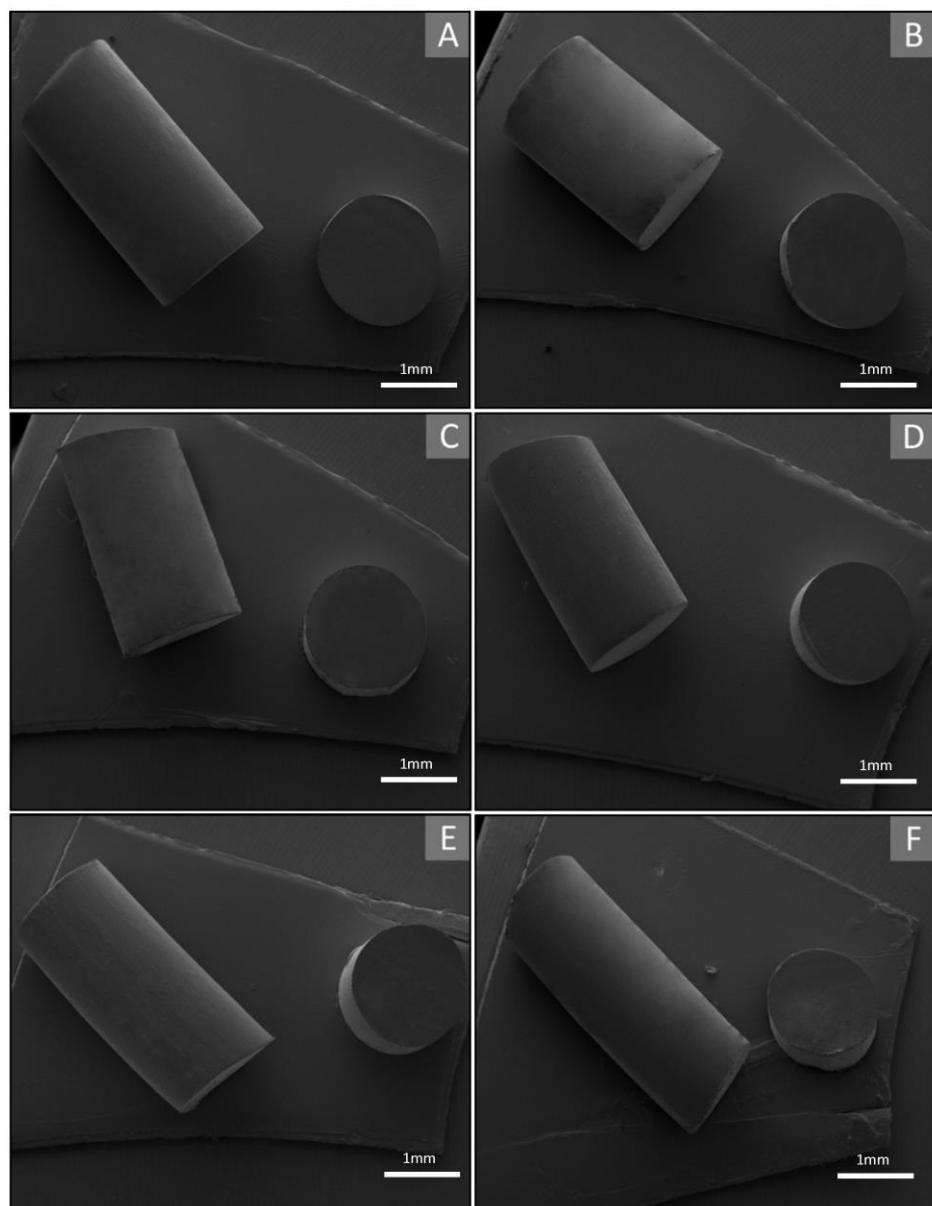
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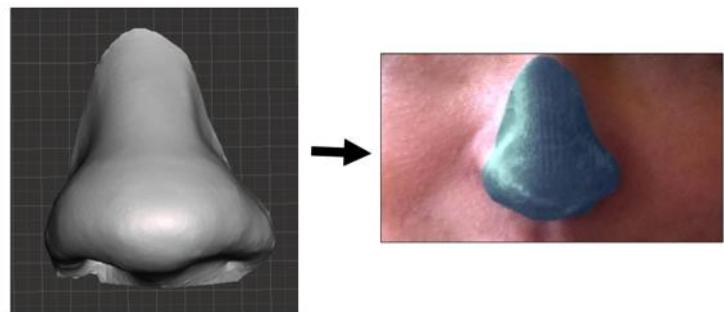
662 **Figure 2**



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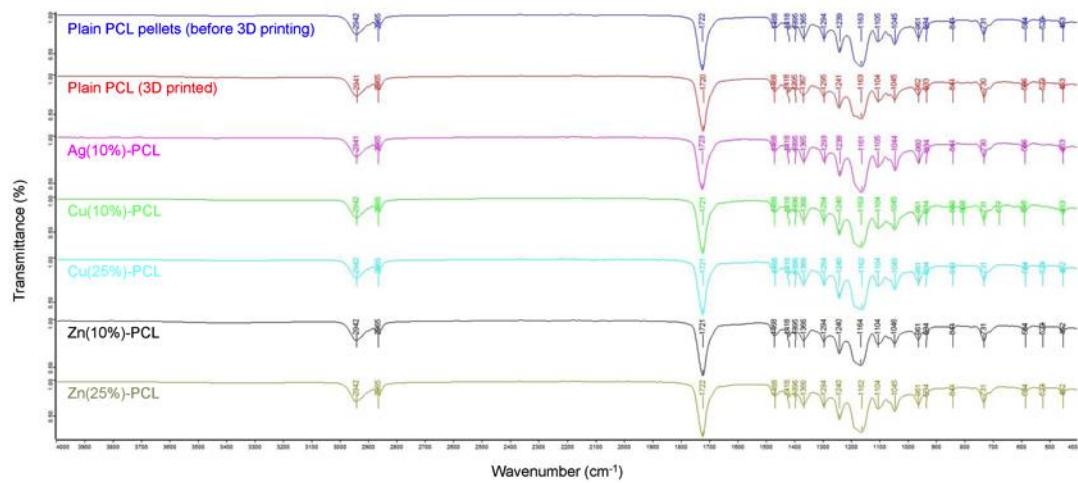
665 **Figure 3**



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668 **Figure 4**

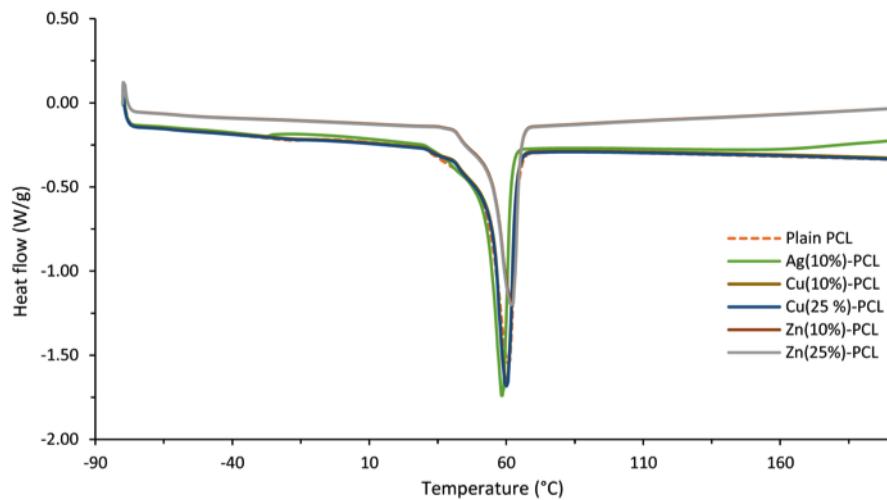


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672 **Figure 5**

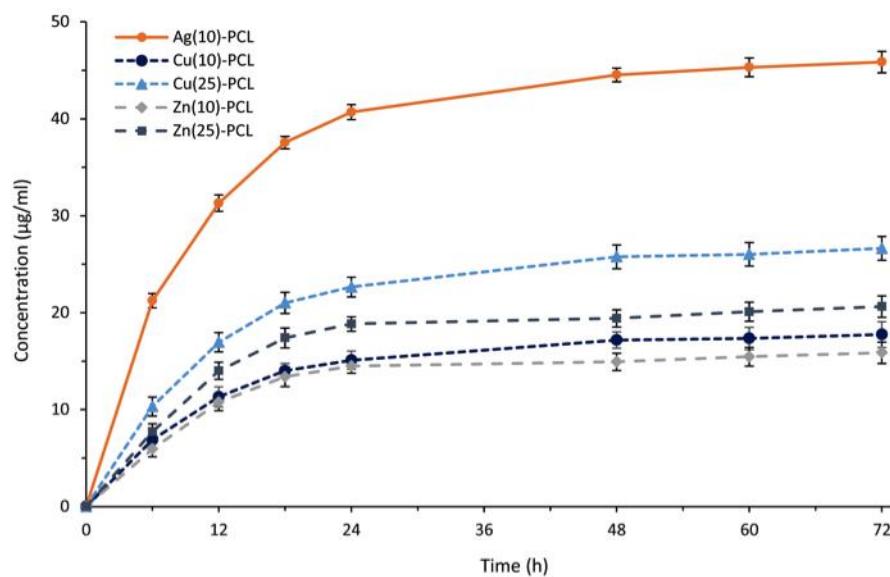


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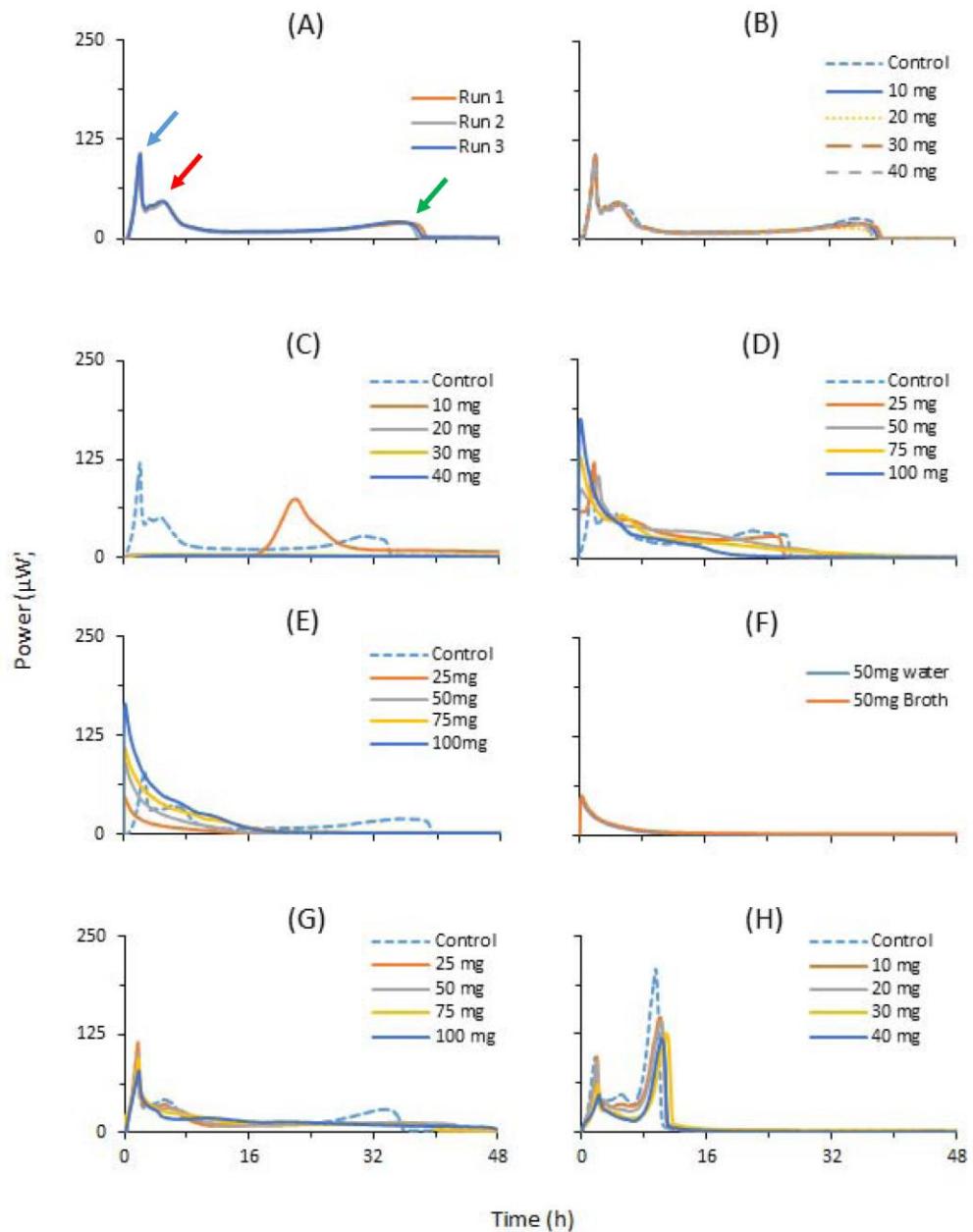
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679 **Figure 7**

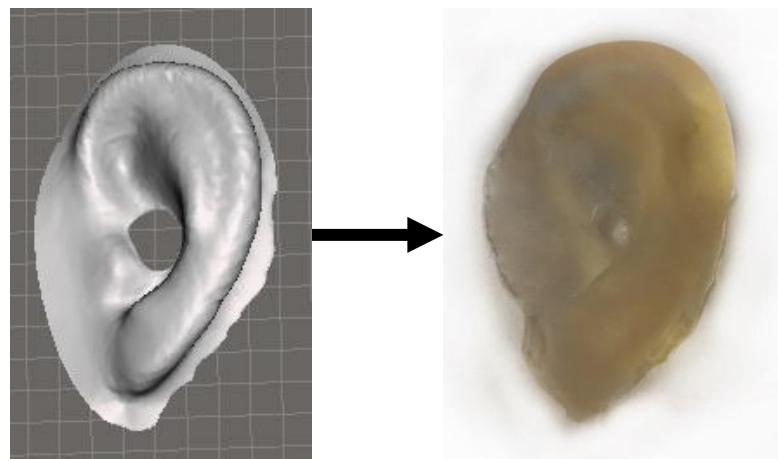


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682 **Appendix 1**

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685 **Figure 8.** 3D scan model of an ear (left) and the printed wound dressing of this model with Ag-PCL (right).