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1 **Electrospun Collagen-Based Nanofibres: A Sustainable Material for Improved**  
2 **Antibiotic Utilisation in Tissue Engineering Applications**

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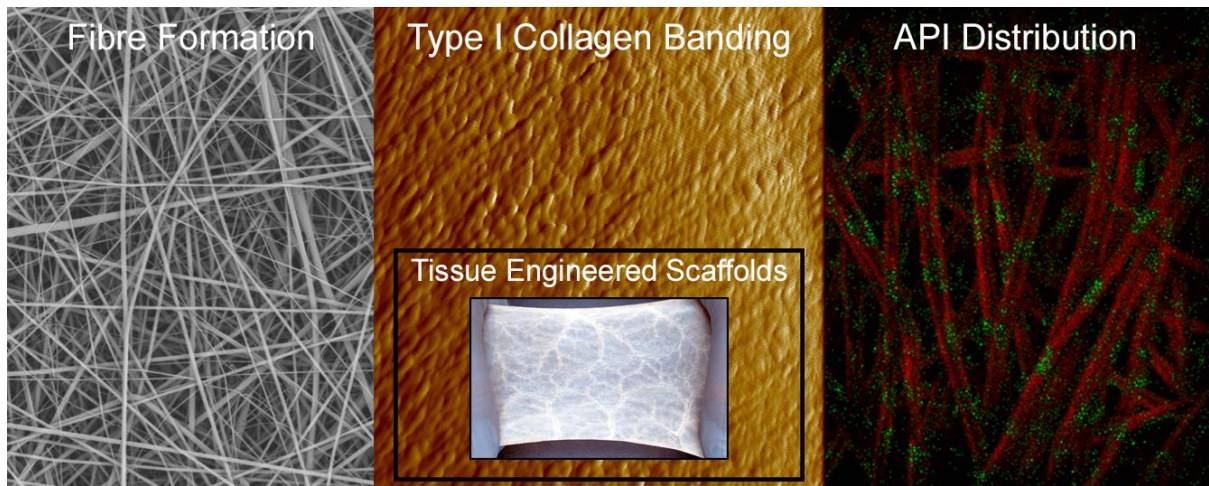
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22

23 **ABSTRACT**

24 For the creation of scaffolds in tissue engineering applications, it is essential to control the  
25 physical morphology of fibres and to choose compositions which do not disturb normal  
26 physiological function. *Collagen*, the most abundant protein in the human body, is a well-  
27 established biopolymer used in electrospinning compositions. It shows high *in-vivo* stability  
28 and is able to maintain a high biomechanical strength over time. In this study, the effects of  
29 collagen type I in polylactic acid-drug electrospun scaffolds for tissue engineering  
30 applications are examined. The samples produced were subsequently characterised using a  
31 range of techniques. Scanning electron microscopy analysis shows that the fibre  
32 morphologies varied across PLA-drug and PLA-collagen-drug samples – the addition of  
33 collagen caused a decrease in average fibre diameter by nearly half, and produced  
34 nanofibres. Atomic force microscopy imaging revealed collagen-banding patterns which  
35 show the successful integration of collagen with PLA. Solid-state characterisation suggested  
36 a chemical interaction between PLA and drug compounds, irgasan and levofloxacin, and the  
37 collagen increased the amorphous regions within the samples. Surface energy analysis of  
38 drug powders showed a higher dispersive surface energy of levofloxacin compared with  
39 irgasan, and contact angle goniometry showed an increase in hydrophobicity in PLA-  
40 collagen-drug samples. The antibacterial studies showed a high efficacy of resistance against  
41 the growth of both *E. coli* and *S. Aureus*, except with PLA-collagen-LEVO which showed a  
42 regrowth of bacteria after 48 h. This can be attributed to the low drug release percentage  
43 incorporated into the nanofibre during the *in vitro* release study. However, the studies did  
44 show that collagen helped shift both drugs into sustained release behaviour. These ideal  
45 modifications to electrospun scaffolds may prove useful in further research regarding the  
46 acceptance of human tissue by inhibiting the potential for bacterial infection.



47

48 **KEYWORDS**

49 Electrospinning, Collagen, Physicochemical Characterisation, Drug Release, Imaging.

50

51 **1. INTRODUCTION**

52 Within the field of tissue engineering, there are a number of different applications that can  
53 be explored relating to the combination of synthetic and natural polymers, and integration  
54 with various active pharmaceutical ingredients. For example, surgical wound closure  
55 involves the bringing together of opposing surfaces using glues, staples and/or sutures. The  
56 re-joined tissues then undergo a primary hyper-proliferative stage, characterised by clot  
57 formation and the recruitment of inflammatory cells (macrophages) into the wound<sup>1</sup>.  
58 Secretion of local tissue mediators encourages cell migration and begins the process of scar  
59 formation<sup>2</sup>. Wound contraction, a result of tissue remodelling, increases the tensile strength  
60 of skin across lesions and increases (or decreases) the risk of ruptures. During the healing  
61 stage, the tissue is open to infection itself and as a pathway into deeper tissue structures.  
62 Infection can result in significant complications (e.g. prolonged systemic antibiotic  
63 administration, re-operation for debridement) for the patient in the short and long-term. In  
64 supporting healing, the purpose of an added matrix task may be to provide tensile strength,  
65 to encourage controlled epithelialisation and new vascular growth and to decrease the  
66 formation of bacterial biofilms. The fabrication of scaffolds for wound repair has become  
67 important, especially the formation of tissue-specific scaffolds<sup>3</sup>.

68 For the creation of scaffolds it is essential to mimic the chemical composition, the physical  
69 morphology, and the biological functions of the human body<sup>4</sup>. Scaffolds can be created  
70 using synthetic polymers (e.g. polycaprolactone) or natural polymers (e.g. chitosan), or a  
71 combination of both – the addition of natural polymers can be highly advantageous as these  
72 may avoid the stimulation of chronic inflammation, immunological reactions and toxicity<sup>5</sup>.  
73 An example of a natural polymer is *collagen*, which is the most abundant protein in the

74 human body, a key element of the extracellular matrix (ECM), and imparts structural  
75 integrity and tensile strength to tissues<sup>6</sup>. Using collagen in scaffolds has been previously  
76 shown to show a high in vivo stability and is able to maintain a high biomechanical strength  
77 over time<sup>7</sup>.

78 One method that is commonly used to fabricate scaffolds for tissue engineering is  
79 *electrospinning*. The technique utilises electrostatic forces to stretch a dilute polymer  
80 solution as it solidifies<sup>8</sup>. It is an ideal micro- and nanofibre fabrication technique for tissue  
81 engineering, as the fibres within the resulting scaffold closely mimic the size and structure of  
82 the native extracellular matrix<sup>9</sup> and the use of solvent and aqueous based systems are  
83 useful for the integration of biological compounds, such as collagen. Various practices of  
84 electrospinning collagen, including collagen-elastin blends<sup>10</sup>, collagen-polycaprolactone  
85 scaffolds for vascular tissue engineering<sup>11</sup>, and fibrinogen fibres which allows native  
86 deposits of collagen during cell growth<sup>12</sup> have previously been studied. Despite these  
87 examples of electrospinning involving collagen, there is an apparent gap in the research  
88 concerning the integration of drugs in these electrospun scaffolds. The integration of drugs,  
89 such as antibiotics, with polymer-collagen blends may be critical in the future success of  
90 human tissue accepting the scaffolds; there are many clinical applications for scaffolds that  
91 require controlling growth of bacteria. For example, one of the requirements for controlling  
92 intra-abdominal infection is to maintain satisfactory levels of an antimicrobial drug during  
93 drug administration<sup>13</sup>. In this regard, the sustained release of drugs is critical to the success  
94 of the device. Sustained release of drugs with electrospun materials have been previously  
95 studied, including polylactic acid with diclofenac sodium (an anti-inflammatory agent)<sup>14</sup>,  
96 metronidazole-loaded polycaprolactone nanofibres<sup>15</sup>, and polyvinylidene fluoride with

97 enrofloxacin (antibiotic)<sup>16</sup>. Given the range of success there has been with electrospinning  
98 polymer with drug, and polymer-collagen blends, the next area of research should be to  
99 determine whether we can alter any of the characteristics of scaffolds with the inclusion of  
100 collagen, whether it be mechanical, chemical or drug release changes.

101 The purpose of this study is to examine the physicochemical properties, bacterial response,  
102 drug loading and bio-functionalisation of electrospun scaffolds that have been prepared  
103 using Type I Collagen with a supporting synthetic polymer. The polymer chosen for this  
104 study is *Polylactic-acid* (PLA), a biodegradable and biocompatible polymer. It has been  
105 approved by the US Food & Drug Administration for safe clinical use as resorbable sutures<sup>17</sup>.  
106 PLA has also been used clinically for foot fractures (PLA pins)<sup>18</sup>, temporary implants<sup>19</sup> and  
107 drug delivery carriers<sup>20</sup>. In this study, solutions were modified by the addition of type I  
108 collagen. The matrix was loaded and electrospun with two drugs, *triclosan* (an antibacterial  
109 agent used commonly in soaps, detergents and surgical cleaning agents) or *levofloxacin* (a  
110 broad-spectrum quinolone antibiotic used commonly in clinical practice to treat  
111 gastrointestinal infections). The electrospun fibres were then characterised through various  
112 methods: the morphology of the electrospun fibres was characterised by scanning electron  
113 microscope (SEM) and Atomic Force Microscope (AFM), solid state characterisation was  
114 performed by x-ray powder diffraction (XRPD) and differential scanning calorimetry (DSC),  
115 surface characterisation of the two drugs was investigated by surface energy analysis (SEA)  
116 and contact angle goniometry (CAG), and drug efficacy (e.g. *in vitro* release studies,  
117 antibacterial studies and time-of-flight secondary ion mass spectrometry (ToF-SIMS)). The  
118 results from this study should help to build a profile of data in order to aid future work with  
119 regards to tissue engineering research specific to the controlled release of drugs.



## 120 2. MATERIALS & METHODS

### 121 2.1 Materials

122 Polylactic acid (PLA) with a mixed molecular weight was the chosen polymer for this study  
123 and was used as purchased from the suppliers (Sigma-Aldrich, *GF45989881*), and the  
124 collagen used was type I from calf skin (Sigma-Aldrich, *C9791*). Irgasan (5-Chloro-2-(2,4-  
125 dichlorophenoxy)phenol), Triclosan, >97%, (Sigma-Aldrich, *72779*) and levofloxacin ((*S*)-9-  
126 fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-*de*]-1,4-  
127 benzoxazine-6-carboxylic acid) >98%, (Sigma-Aldrich, *28266*) were obtained commercially.  
128 The solvents used for the electrospinning were also commercially purchased from Sigma-  
129 Aldrich, specifically chloroform (anhydrous, containing amylenes as stabilizers grade, >99%),  
130 N,N-dimethylformamide (DMF, anhydrous grade 99.8%) and 1,1,1,3,3,3-hexafluoro-2-  
131 propanol, (GC grade >99%).

### 132 2.2 Preparation of PLA Solutions

133 Investigations of the polymer-drug solutions prepared showed that the optimum w/w  
134 concentration for electrospinning was 8%. This particular concentration was used as  
135 previous experimentation revealed that higher concentrations did not result in suitable fibre  
136 formation (suitable meaning regular fibre diameters and no signs of beading, data not  
137 shown). PLA (8% w/w), and a 9:1 (w/w) ratio of chloroform (CLF) to N,N-dimethylformamide  
138 (DMF). For the formulations containing collagen type I, both polymers were solubilised in  
139 hexafluoropropanol (HFP) with a total polymer (collagen + PLA) concentration of 8%. PLA  
140 was blended with collagen containing 1% collagen ( $M_{\text{PLA}} / [M_{\text{Collagen}} + M_{\text{PLA}}] \times 100$ )<sup>21</sup>. Collagen  
141 at a 1% concentration was used as higher concentrations, in the range 2% to 10% w/w,  
142 yielded unstable and inconsistent fibre formations.

143 The solutions were mixed through 30 min in the centrifuge, a further 30 min in a sonicator  
144 and a final 1 h with a magnetic stirrer, which resulted in a solution that appeared  
145 homogeneous and composed of a single phase. The solution was left overnight, and a  
146 further 30 min of sonication was applied the following morning in order to confirm the  
147 uniformity of the solution. For the irgasan-loaded solutions, the same method was applied,  
148 at a concentration of 1% irgasan w/w. The concentration of the levofloxacin-loaded  
149 solutions was adjusted to 0.5% w/w to facilitate accurate measurement of the drug release  
150 profile. All the solutions remained clear after preparation. This process was based on the  
151 method used by Hall Barrientos *et al* (2016) for solution preparation<sup>22</sup>.

### 152 **2.3 Electrospinning**

153 Various scaffolds were fabricated for each polymeric solution, using an in-house  
154 electrospinning apparatus which consisted of a syringe pump (Harvard Apparatus PHD 2000  
155 infusion, US) and two 30kV high-voltage power supplies (Alpha III series, Brandenburg, UK).  
156 The polymer solution was loaded into a 5 mL glass syringe and fed through tubing with a  
157 metal needle tip attached at the end. The needle was clamped into place, to allow for a  
158 high-voltage supply to run through it – this allowed for an electric field to be created  
159 between the needle and the target plate. The syringe was clamped to a pump, which  
160 determined the specific injection flow rate of the polymeric solutions. For each of the three  
161 solutions (e.g. unloaded, irgasan loaded, and levofloxacin loaded), 3 varying flow rates of  
162 0.5, 1 and 1.5 mL h<sup>-1</sup> were applied across varying voltages of 2 kV – 5 kV (across the needles)  
163 and 10 kV – 18 kV<sup>22</sup>. A 21 gauge needle, at a deposition distance of between 10 – 12 cm was  
164 used. The process was performed at ambient room temperature (approximately 21°C) with  
165 a relative humidity of between 2 – 4 % (electrospinning was not performed is the relative

166 humidity was greater than 5%). The solution was electrospun onto the target that was  
167 covered with aluminium foil, in order for the final material to be removed and used for  
168 further characterisation.

#### 169 **2.4 Scanning Electron Microscopy**

170 A scanning electron microscope (SEM) was used to determine the morphology and  
171 individual fibre diameter of the electrospun PLA solutions (PLA, PLA-IRG, PLA-LEVO, PLA-  
172 Collagen, PLA-Collagen-IRG, and PLA-Collagen-LEVO). A Hitachi TM-1000 SEM was used to  
173 analyse various samples, in which the samples were mounted on an aluminium plate with  
174 conductive tape. Images of fibres were taken at various locations of each electrospun PLA  
175 scaffold in order to determine the overall uniformity of fibres (n=5). Prior to imaging, the  
176 samples were sputter coated with gold for 30 s using a Leica EM ACE200 vacuum coater,  
177 repeated three times in order to increase the conductivity of the samples. The samples were  
178 imaged in secondary electron mode at 5 kV.

#### 179 **2.5 Mathematical analysis of fibre diameters**

180 Statistical comparisons of samples were conducted using SPSS 20.0 software package (SPSS  
181 Inc., Chicago IL, USA). Descriptive statistics, analysis of variance, Scheffé post hoc test and  
182 Kolgomorov-Smirnov test were performed using 50 individual fibre diameters of each  
183 samples obtained from the morphological analysis.

#### 184 **2.6 Atomic Force Microscopy**

185 Further morphological analysis was undertaken through atomic force microscopy (AFM). A  
186 Multimode 8 microscope (Bruker, USA), with Scanasyst-Air probes (Bruker, USA) was used in  
187 Peak Force Quantitative Nano Mechanics (QNM) mode<sup>23</sup>. The imaging of the fibres was  
188 performed under ambient conditions, with a silicon cantilever probe (Bruker). The tip radius

189 of the probe and the spring constant were calculated to be in the regions of 0.964 nm (18°  
190 tip half angle) and 0.3947 N/m, respectively. The scan sizes ranged from 200 nm to 25 µm,  
191 at a scan rate of 0.977 Hz with 512-sample resolution. The Roughness Average (Ra) values  
192 were determined by entering surface scanning data, and digital levelling algorithm values  
193 were determined using Nanoscope Analysis software V1.40 (Bruker USA). AFM images were  
194 collected from two different samples and at random spot surface sampling.

### 195 **2.7 Differential Scanning Calorimetry (DSC)**

196 Differential scanning calorimetry (DSC) was carried out using a Mettler Toledo DSC822 in  
197 order to examine the thermal properties of the electrospun PLA scaffolds. First, the  
198 scaffolds were cut, weighed and placed in the DSC specific aluminium discs. The disc was  
199 then sealed using a press and subsequently placed in the DSC instrument. The parameters  
200 for DSC analysis were set (parameters are detailed below) via the computer software. The  
201 analysis was then run which took approximately 20 min. The method included heating from  
202 25 °C to 220 °C at 10 °C steps, using standard 40 µl aluminium discs, and each sample  
203 consisted of 4 mg.

### 204 **2.8 X-Ray Powder Diffraction (XRPD)**

205 XRPD was performed in a Bruker D2 Phaser machine and measurements were taken under  
206 CuK $\alpha$  radiation ( $\lambda= 1.5406 \text{ \AA}$ ), 40 kV and 30 mA as X-ray source with K $\beta$  (Ni) filter. Diffraction  
207 patterns were collected with  $2\theta$  ranging from 3° to 70°.

### 208 **2.9 Contact Angle Goniometry (CAG)**

209 To monitor changes in wettability of the scaffolds, sessile drop contact angle for distilled  
210 water was measured by contact angle goniometry, using a contact angle goniometer (Kruss  
211 G30, Germany)<sup>24</sup>.

## 212 **2.10 Surface Energy Analysis**

213 The dispersive surface energy of the raw drug samples was determined by inverse gas  
214 chromatography using a SEA-IGC (Surface Measurement Systems). The samples were  
215 packed into 30 cm (3 mm inside diameter) silanised glass columns, plugged at either end by  
216 silanised glass wool. Various dispersive probes were used; undecane, decane, nonane,  
217 octane, heptane, and hexane were injected at a range of fractional surface coverage in  
218 order to determine the concentration free dispersive surface energy<sup>25</sup>.

## 219 **2.11 <sup>1</sup>H NMR Studies**

220 NMR spectra were recorded on a Varian 600 MHz spectrometer at 298.2 ±0.1 K with  
221 CDCl<sub>3</sub>:DMF (N,N-dimethylformamide), 95:5, V/V% as solvent, using tetramethylsilane (TMS)  
222 as the chemical shift reference compound. The sample volume was 600 µL. <sup>1</sup>H NMR spectra  
223 were recorded with the pulse and acquire sequence (number of transients=16, number of  
224 points=16384, acquisition time=851.968 ms, relaxation delay=1.5 s).

## 225 **2.12 *In Vitro* Drug Release Studies**

226 The drug releases of the irgasan/levofloxacin loaded PLA-blend scaffolds were measured in  
227 order to determine the release profile for each drug. Buffer solutions consisting of  
228 phosphate buffer solution (PBS, pH 7.4) was mixed with 1% sodium dodecyl sulphate (SDS, a  
229 surfactant was used due to the hydrophobic nature of irgasan). The UV absorbance of both  
230 drugs were measured (irgasan at 280 nm<sup>26</sup>, and levofloxacin at 293 nm<sup>27</sup>, respectively) at  
231 various time points (e.g. 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and then every day up to  
232 192 h). For every measurement 3 ml of solution was taken from the vial (consisting of total 6  
233 ml PBS) and replaced with fresh 3 ml buffer in order to satisfy the perfect-sink conditions.  
234 The samples were stored in a temperature room of 37 °C.

235 **2.13 Antibacterial Studies**

236 The antibacterial efficacy of the drug loaded electrospun scaffolds were tested with  
237 *Escherichia coli* (*E. coli*) 8739 and *Staphylococcus aureus* (*S. aureus*) 29213, bacterial strains  
238 representative of gram-negative and gram-positive bacteria, respectively. For this study, a  
239 disc agar diffusion method was used. Luria-Bertani (LB) agar was prepared with the LB  
240 medium consisting of 5 g tryptone, 2.5 g yeast extract, 5 g NaCl and 475 ml of deionized  
241 water. The *E. coli* and *S. aureus* cultures were grown overnight in 5 ml of solution, with both  
242 bacteria inoculated from a single colony. 150  $\mu$ L of the *E. coli* and *S. aureus* cultures were  
243 spread onto plates of LB agar. 6 plates received an *E. coli* inoculum, and the other 6 plates  
244 contained the *S. aureus* inoculum, before adding small pieces of scaffold onto each plate.  
245 Scaffolds containing no drug and no scaffold presence acted as controls. Each scaffold was  
246 placed in triplicate to ensure accurate results. The plates were left for incubation for 48 h,  
247 and subsequently examined at 24 h and 48 h. Diameters of zones in which there was no  
248 bacterial growth were measured, and these were compared across the various drugs and  
249 bacterial strains.

250 **2.14 Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS)**

251 The analyses were carried out with a ToF-SIMS V instrument (ION-TOF GmbH, Münster,  
252 Germany) equipped with a Bismuth cluster Liquid Metal Ion Gun (LMIG). To overcome  
253 charge build-up on the isolative samples, a low-energy electron beam (21 eV) flood gun was  
254 employed and the sample surface potential was optimised for each analysis.

255 **Spectrometry** mode was applied to acquire reference spectra of Levofloxacin, Irgasan and  
256 PLA. Data was recorded in three replicates for both polarities and each acquisition was  
257 made from different areas of the samples used in this study, over a field of view of 100  $\mu$ m  $\times$

258 100  $\mu\text{m}$ . The 30 kV  $\text{Bi}_3^+$  primary ion beam was optimised to deliver a final primary ion dose  
259 density (PIDD) of  $8.0 \times 10^{12}$  (primary ions/ $\text{cm}^2$ ) for each spectrum.

260 **High lateral resolution images** of the four drug-loaded samples were collected over a  
261 surface area of  $50 \mu\text{m} \times 50 \mu\text{m}$ , with a resolution of  $512 \times 512$  pixels (pixel width was circa  $0.1$   
262  $\mu\text{m}$ ), in the negative secondary ion polarity, with a final PIDD equal to  $8.0 \times 10^{12}$  primary  
263 ions/ $\text{cm}^2$ . The images were then processed using SurfaceLab 6.7 software (ION-TOF,  
264 Münster, Germany). All the mass spectral information was recorded in the  $m/z$  range of 0 -  
265 900 Da.

### 266 3. RESULTS

#### 267 3.1 Intermolecular Interactions

268 Preliminary  $^1\text{H}$  NMR study performed in liquid phase suggests the absence of any specific  
269 intermolecular interaction between PLA and LEVO. The  $^1\text{H}$  NMR spectrum of polylactic  
270 revealed two well-defined peaks centered at 5.10 and 1.52 (ppm) with smaller peaks  
271 residing in the vicinity of the main signal (less than 2% in total peak area based on Lorentz  
272 peak fitting) as depicted in Figure 1. The relatively sharp (2-3 Hz half width) and uniform  
273 quartet and doublet signals of polylactic acid indicate a well-defined conformation  
274 throughout the polymer chain, and albeit the varying chain length the chemical  
275 environment of most monomer units is implied to be highly similar allowing for high  
276 mobility on account of the narrow peaks, i.e. high T2. The peaks at 7.26, 7.94, 2.89, and 2.81  
277 are that of  $\text{CHCl}_3$ , the formyl hydrogen of DMF, and the two methyl peaks of DMF,  
278 respectively. The  $^1\text{H}$  NMR spectrum of polylactic acid doped with 0.1% levofloxacin showed  
279 no difference in the NMR parameters of the polymer; the peaks of levofloxacin were  
280 detected at the expected chemical shift ranges, however these signals appeared with too

281 low SNR to be useful for further measurements. It can only be inferred from the NMR  
282 measurements that a possible presence of intermolecular interaction between polylactic  
283 acid and levofloxacin does not bring about detectable changes in the  $^1\text{H}$  NMR spectra.

### 284 3.2 Fibre Morphology

285 A one-way between-groups analysis of variance (ANOVA) indicated that there are significant  
286 differences ( $p < 0.001$ ) between the investigated formulations. Applying Scheffé post-hoc  
287 comparison, significant differences were confirmed between samples except for PLA  
288 compared with PLA-IRG and PLA-Collagen compared with PLA-Collagen-IRG. The latter was  
289 also corroborated by the overlap between the obtained confidence intervals; whilst in case  
290 of the other samples the confidence intervals are separated from each other. Except for  
291 PLA-Collagen-LEVO, 99.7 % of the measured fibre diameters fall between the range of mean  
292  $\pm 3$  SD, and similarities of the mean and median values suggest that these samples are  
293 normally distributed (Figure 2).

294 Fibre diameter histograms were assayed using Kolmogorov-Smirnov test ( $p < 0.01$ ) of equality  
295 of distributions (Table 2), which indicated that there is no significant difference, when PLA  
296 compared with PLA-IRG; and PLA-Collagen compared with PLA-Collagen-IRG. On the other  
297 hand, distributions of PLA/PLA-IRG; PLA-Collagen/PLA-Collagen-IRG; PLA-LEVO; PLA-  
298 Collagen-LEVO were significantly different when compared with each other. It must be  
299 noted that PLA-Collagen-LEVO exhibited the highest coefficient of variation (72.7 %), whilst  
300 PLA-LEVO had the lowest value (17.2 %) (Table 1).

301 The SEM images (Figure 3) demonstrated a range of morphologies across both polymer-drug  
302 and polymer-collagen-drug electrospun scaffolds. The PLA-drug scaffolds (Figure 3a – 3c)



303 showed consistent fibre diameter and the addition of IRG and LEVO caused very slight  
304 beading within the fibres.

305 The AFM images (Figure 4) show the morphology of the fibres at a greater detail and  
306 resolution. PLA-drug scaffolds (Figure 4a – 4b) show a slight variation of morphology across  
307 their surfaces; PLA fibres have a relatively smooth surface with what appears to be beading  
308 (possible solvent residue) on the surface, PLA-IRG fibres generally showed smooth  
309 morphology with no signs of active pharmaceutical ingredient (API) or solvent residue on  
310 the surface, whereas the PLA-LEVO fibres showed a rougher morphology which may have  
311 resulted from crystals of the drug at the surface – this observation was supported by the  
312 release profile (see: Results, Drug Efficacy). The surface morphology for the PLA-collagen-  
313 drug scaffolds (Figure 4d – 4f) showed the presence of collagen “wrapping” the polymeric  
314 fibres in a helical manner. This morphology was consistent throughout, which may highlight  
315 a helical coating of the collagen through the fabricated scaffolds. There appeared to be no  
316 presence of drug on the surface of these fibres.

### 317 **3.3 Solid State Characterisation**

318 The graphs in figure 4 indicate the various DSC isotherms for PLA-drug fibres (Figure 5a), and  
319 PLA-Collagen fibres (Figure 5b). For most of the samples, the glass transition temperature  
320 ( $T_g$ ) is observed around 60°C, the crystallisation temperature ( $T_c$ ), an exothermic peak, is  
321 observed around 90 - 115°C and the melting point, an endothermic peak, is observed at  
322 ~145°C. Overall the  $T_c$  for PLA-drug samples is lower than the PLA-collagen-drug samples. In  
323 the case of PLA-IRG (Figure 5a), the  $T_g$  appears to be suppressed and a  $T_c$  was not measured  
324 at all.

325 XRPD data shown in figure 5 indicate that PLA fibres are semi-crystalline, indicated by broad  
326 region, followed by a sharp peak at  $\sim 17^\circ$ . It can be observed in **Figure 6a** that the addition of  
327 IRG causes an increase in the crystallinity of the polymer-drug sample, due to the sharp  
328 polymer peak at  $\sim 17^\circ$ . There are no significant peaks arising within the PLA-IRG graph  
329 related to the IRG intensity. The PLA-collagen-IRG graph indicates that the sample is in an  
330 amorphous state, due to the very broad peak observed from  $\sim 5^\circ$  to  $\sim 25^\circ$ . The graphs  
331 relating to the PLA-LEVO sample (figure 5b) show two significant peaks at  $\sim 7^\circ$  (relating  
332 levofloxacin) and  $\sim 17^\circ$  (relating to PLA). Again, the addition of collagen to these samples  
333 resulted in a broad amorphous peak ranging from  $\sim 5^\circ$  to  $\sim 25^\circ$ .

### 334 **3.4 Surface Analysis**

335 The dispersive surface energy of powder irgasan was measured at  $37.32 \text{ mJ/m}^2$ , and a BET  
336 specific surface area of  $0.0103 \text{ m}^2/\text{g}$ . Levofloxacin had a higher dispersive surface energy of  
337  $47.34 \text{ mJ/m}^2$  and a BET specific surface area of  $0.0481 \text{ m}^2/\text{g}$ .

338 The contact angle measurements shown in Figure 7 highlight the differences across PLA-  
339 drug and PLA-collagen-drug combinations; PLA-LEVO had a lower overall starting and  
340 finishing contact angle ( $108^\circ \pm 2.11$  to  $50^\circ \pm 2.35$ , respectively), compared with the PLA and  
341 PLA-IRG ( $120^\circ \pm 1.09$  to  $80^\circ \pm 1.22$ , respectively). The samples containing collagen had a  
342 greater starting contact angle ( $130^\circ \pm 3.42$ ) and final contact angle reading ( $120^\circ \pm 4.12$ ).

### 343 **3.4 Drug Efficacy and Antibacterial Studies**

344 The cumulative drug release profiles are presented in Figure 8; both PLA-IRG and PLA-  
345 collagen-IRG samples exhibited sustained release behaviour over 192 h, with a final release  
346 of  $\sim 40\%$ . PLA-LEVO exhibited burst release behaviour, releasing  $\sim 20\%$  within the first 24 h of  
347 measurement. PLA-collagen-LEVO showed a significantly lower release percentage initially,

348 however it appeared to exhibit sustained release behaviour, with a final cumulative release  
349 of 25%. The total drug content was determined by  $\text{Concentration} \times \text{Volume of Medium} \times$   
350  $\text{Dilution Factor} / 1000$  (correction factor). Therefore,  $\% \text{ Drug Release} = \text{Amount of Drug} /$   
351  $\text{Theoretical Drug Amount} \times 100$ .

352 Antibacterial measurements were taken at 24 h and 48 h (Figure 9, 48 h only). The PLA-drug  
353 samples show a high efficacy to control the growth of both *E. coli* and *S. aureus*. In  
354 particular, PLA-LEVO (2.1 cm) had a higher average inhibition zone than PLA-IRG (1.0 cm).  
355 PLA-collagen-IRG resisted both strains of bacteria in a similar manner to the PLA-IRG sample  
356 (average inhibition zone = 1.1 cm). Finally, the PLA-collagen-LEVO sample showed an  
357 average inhibition zone to *E. coli* of 1.0 cm; however white spores of bacteria were re-  
358 forming near the sample. This sample also had a smaller efficacy with inhibiting the growth  
359 of *S. aureus*, demonstrating an average inhibition zone of 0.4 cm.

### 360 3.5 ToF-SIMS

361 Imaging data showed a difference in the distribution of the APIs in the various strands. The  
362 compounds of interest are identified by unique characteristic ion peaks; PLA at  $m/z$  71  
363 ( $[C_3H_3O_2]^-$ ) and  $m/z$  89 ( $[C_3H_5O_3]^-$ ), Levofloxacin at  $m/z$  360 ( $[C_{18}H_{19}FN_3O_4]^-$ ,  $[M-H]^-$ ) and  $m/z$   
364 316 ( $[C_{17}H_{19}FN_3O_2]^-$ ), and Irgasan at  $m/z$  287 ( $[C_{12}H_6^{35}Cl_3O_2]^-$   $[M-H]^-$ ), and isotopes  $m/z$  289  
365 ( $[C_{12}H_6^{35}Cl_2^{37}ClO_2]^-$ ) and  $m/z$  291 ( $[C_{12}H_6^{35}Cl^{37}Cl_2O_2]^-$ ). The total ion images and the overlays  
366 of single ion images for the characteristic peaks of PLA, PLA-collagen and the two drugs are  
367 reported in Figure 10. With regards to the PLA-IRG and PLA-LEVO electrospun scaffolds, by  
368 the ion images Irgasan appears to be homogeneously distributed throughout the sample,  
369 whilst the presence of Levofloxacin is concentrated in multiple small regions on the surface

370 of the fibers. This confirms the data obtained with the AFM analysis and with the release  
371 study – (see: Results, Fibre Morphology and Results, Drug Efficacy).

372 The diagnostic peaks for both the APIs in the PLA-collagen-API samples present normalized  
373 intensities which are 2 to 3 times lower than the corresponding peaks in the PLA-API  
374 samples, making it difficult to comment on their distribution. The reduced signal could be  
375 caused by matrix effects, an artefact of ionization. Here, it is possible that the presence of  
376 collagen in the system suppressed the ionization of the APIs. Alternatively, it could suggest  
377 a lower concentration of the APIs present on the surface of the PLA-Collagen fibres  
378 compared to the PLA ones, and could be interpreted as a better embedding of the APIs into  
379 the strands.

## 380 **4. DISCUSSION**

### 381 **4.1 Fibre Morphology**

382 The differences in fibre form between PLA-drug and PLA-collagen-drug samples can be  
383 clearly seen in figures 2 and 3, where the addition of collagen has created smaller diameters  
384 of the fibres. This is due to the nature of collagen fibre formation, which usually form  
385 nanofibres of around 100 nm<sup>28</sup>. Although, the fibres created in this study are significantly  
386 bigger than other studies (e.g. average collagen fibres diameters = ~400 nm<sup>10</sup>) this may be  
387 largely due to the blend of PLA and collagen – with PLA-drug solutions producing fibres with  
388 a diameter greater than 2 µm. Fibre formation may also be more effective with collagen,  
389 due to the HFP solvent; the low boiling point (58.2 °C) of this solvent allows for a quicker  
390 evaporation during the electrospinning process, which in turn means that the fibres are  
391 being deposited in a dry state. HFP is also a denaturing organic solvent, and therefore the  
392 interaction between solvent and collagen may cause change in the structure of the

393 proteins<sup>29</sup>. When compared to polycaprolactone fibres (as conducted in our previous study)  
394 electrospun in similar conditions, the addition of levofloxacin increased the overall average  
395 fibre diameter (PCL =  $\sim 1.83 \mu\text{m}$ <sup>8</sup> PCL-LEVO =  $\sim 2.8 \mu\text{m}$ <sup>22</sup>).

396 It was also observed that the addition of levofloxacin within both PLA and PLA-collagen  
397 blends resulted in a decreased average fibre diameter; this can be linked to the rheological  
398 behaviour of the solutions where levofloxacin increased the viscosity of the solution, which  
399 normally results in a change in fibre size<sup>30</sup>. There also appears to be evidence of levofloxacin  
400 appearing at the surface of the fibres (figure 4c), however this may be expected for this  
401 particular drug due to the high dispersive surface energy (see Results: Surface Analysis). If  
402 the drug has a high surface energy, this affects the viscosity of the solution, which in turn  
403 alters the surface tension.

404 The AFM images of the polymer-collagen blends revealed a helical pattern of collagen  
405 around the fibres – the fibrils of collagen exhibit a repeating, nano-banding pattern.  
406 Collagen naturally forms a coiled structure, and the underlying alpha chains within the  
407 collagen fibrils could be responsible for the repeating banding pattern observed<sup>31</sup>. This may  
408 be useful within a tissue engineering context, given that this repeat banding is thought to  
409 expose a binding site in the native collagen fibril that enhances cell adhesion and migration<sup>6</sup>.

#### 410 **4.2 Solid State Characterisation**

411 The DSC data was vital in understanding any potential chemical interactions between the  
412 drug and polymer. In figure 5, the PLA-IRG plot, shows that there is no visible exothermic  
413 reaction ( $T_c$ ) occurring after the initial glass transition phase, and also no indication of the  $T_m$   
414 of the raw IRG drug at around  $60^\circ\text{C}$  – this can be interpreted as the IRG being fully integrated  
415 within the polymeric structure<sup>32</sup>. The  $T_c$  values recorded were greater in the samples

416 containing collagen which may be attributed to dehydration of the collagen<sup>33</sup>. This may be  
417 indicative of the collagen successfully embedding or interacting with the polymer. A  
418 reduction in the  $T_g$  at around 60 °C of the PLA-Collagen-LEVO can be observed, which again  
419 may indicate that there is no presence of free LEVO particle in this sample – this is perhaps  
420 why a sustained release behaviour is observed in section 4.4 (drug efficacy).

421 The data presented by the DSC was effectively confirmed through the XRPD data (figure 6).  
422 The PLA-IRG sample can be seen to remain in a near semi-crystalline form (slight broad  
423 peak, followed by sharp crystalline PLA peak), with no IRG peaks. It can be concluded from  
424 this that the IRG is fully embedded within the polymeric network. Similarly, no IRG peaks  
425 were visible within the PLA-collagen-IRG graph; however, the broad peak does indicate that  
426 the material is in an amorphous form. The PLA-LEVO graph showed a peak, indicating that  
427 the drug is still in crystalline form and is not incorporated uniformly into the polymeric  
428 network. A similar amorphous peak was observed in the PLA-collagen-LEVO peak, which  
429 may indicate a better encapsulation of drug.

### 430 **4.3 Surface Analysis**

431 Understanding the surface energy of the drug molecules was an important part of this  
432 study, since it has successfully helped to understand the reasons behind particular  
433 behaviour relating to fibre morphology and overall drug encapsulation. The surface energy  
434 analysis of the two drugs indicated a higher dispersive surface energy for levofloxacin  
435 compared with irgasan. Given that IRG and LEVO are both in a crystalline form, and that  
436 polylactic acid is naturally semi-crystalline, there may be an increased possibility of the two  
437 drugs integrating into the polymeric matrix. The two drugs may require a higher energy in  
438 order to disperse properly within the PLA polymeric structure. With irgasan having a lower

439 dispersive surface energy and a hydrophobic nature, there may be a reduced energy  
440 requirement for this drug to properly disperse within a solvent solution. If levofloxacin has a  
441 higher dispersive surface energy, then the transfer of energy between LEVO and solvent will  
442 be insufficient to overcome the drug-drug attractions. Therefore, not all the levofloxacin  
443 molecules in solution will be surrounded by solvent molecules and dispersed fully into  
444 solution<sup>34</sup>. At equilibrium, hydrophobic molecules preferentially partition into the organic  
445 phase, typically due to a high log P value<sup>35</sup> - the log P of levofloxacin is 1.27<sup>36</sup> and the log P  
446 of Irgasan is 4.76<sup>37</sup>. Based on the positive values on the log P, both drugs are hydrophobic  
447 and Irgasan is more hydrophobic than LEVO. With both drugs and PLA exhibiting a  
448 hydrophobic nature, this suggests that hydrophobic interactions might occur between the  
449 materials. If the hydrophobic interactions are a dominant, this should be more  
450 advantageous in increasing encapsulation efficiency<sup>38</sup>.

451 The change in contact angle may reflect the encapsulation of both drugs within the  
452 polymer matrix. It can be clearly seen in figure 7 that the contact angle was lowest for the  
453 PLA-LEVO sample. This can be attributed to the lower hydrophobic nature of levofloxacin,  
454 the high dispersive surface energy and the probability of the drug on the surface of the  
455 fibres (see Results: Fibre Morphology, AFM). With the PLA-collagen-drug samples, the  
456 contact angles are greater, implying that the samples are more hydrophobic. These results  
457 differ from the contact angle measurements in other studies, in particular collagen is found  
458 to have previously increased the hydrophilicity of PLA samples<sup>39</sup>. It may be the case that the  
459 collagen fibrils have interacted within the polylactic acid based nanofibre. This strong  
460 hydrophobic connection may be linked to the better integration of both irgasan and  
461 levofloxacin.

#### 462 4.4 Drug Efficacy

463 The release profiles of both irgasan and levofloxacin are similar to the results in a previous  
464 study using *polycaprolactone*<sup>22</sup>. However, the final cumulative release percentages with  
465 *Polyactic acid* appear to be lower (Final PCL scaffolds drug release ~50%, PLA scaffolds  
466 ~20% to 40%). This may suggest a poorer drug encapsulation with PLA, which may be due to  
467 the fact that PLA is more hydrophilic than PCL<sup>40</sup>. The hydrophobic interactions between PLA  
468 and IRG may potentially be the cause of the sustained release behaviour *in vitro*, and the  
469 lower hydrophobic nature of LEVO may be an even further reduction in a strong polymer-  
470 drug interaction. As mentioned in the surface analysis section, the higher dispersive surface  
471 energy may cause this weak interaction between PLA and LEVO; AFM images in figure 4  
472 indicating the possible presence of drug at the surface of the fibres. This presence of drug at  
473 the surface would result in a rapid uptake of water, and therefore be the reason behind the  
474 burst release behaviour observed *in vitro*. The addition of type I collagen to the polymer-  
475 drug samples resulted in a difference in release behaviour with LEVO – this drug released in  
476 a sustained release time profile, implying that collagen may be directly affecting the release  
477 of the drug. Literature evidence suggests that the hydrophilic, carboxyl functional groups in  
478 the polymer could be interacting with basic functions expressed by the collagen<sup>41</sup> which in  
479 turn results in a change in the hydrophobicity of the fibre, which may result in the sustained  
480 release time profile for the levofloxacin with the PLA-collagen sample.

481 The antibacterial studies were consistent with the drug release profiles, and indicated that  
482 the formulations were effective in inhibiting the growth of *E. coli* and *S. aureus*. The larger  
483 zones of inhibition can be seen in the PLA-LEVO sample, which exhibits burst release  
484 behaviour. The smaller inhibition zones for PLA-IRG and PLA-collagen-IRG were indicative of



485 the sustained release profiles. The sustained release profile of PLA-collagen-LEVO was also  
486 confirmed, given that there were small areas of bacterial growth – this can be attributed to  
487 the low cumulative release percentage at 48 h of the study by which time < 10% of the drug  
488 had been released).

#### 489 **4.5 ToF-SIMS**

490 The visualisation of the distribution of the APIs on the sample surface contributed to gain a  
491 better understanding about the degree of drug encapsulation and in general about the  
492 interactions between the drugs and the fibres. This result concurs with the suggested  
493 explanation with regards to the release behaviour and is consistent with the results from  
494 the other techniques described in this paper.

#### 495 **5. CONCLUSION**

496 It can be seen that the inclusion of type I collagen significantly altered various characteristics  
497 of electrospun polymer-drug scaffolds. The addition of collagen caused an overall decrease  
498 in fibre morphology and average fibre diameter across both sets of drugs, irgasan and  
499 levofloxacin, with nanofibers forming in the PLA-collagen-LEVO samples. AFM images  
500 revealed collagen fibril banding on the surface of the fibres which suggest there is an  
501 interaction between the polymer-drug and collagen. There were also changes in the solid-  
502 state characteristics of the samples, given that the DSC results indicate an increase in  
503 amorphousness of the polymer-drug-collagen samples, and this was confirmed through  
504 typically broad peaks in the XRPD results. Most interestingly, the surface energies of the  
505 scaffolds were modified with an increase in the hydrophobicity of the polymer-collagen-  
506 drug noted. In particular, the PLA-collagen-LEVO sample showed a high deal of  
507 hydrophobicity, which was unusual given the low hydrophobic nature of LEVO – this could

508 potentially be attributed to the strong hydrophobic interactions between the PLA and the  
509 hydrophobic banding in collagen fibrils, resulting in better encapsulation of the drug. Finally,  
510 the drug release profiles of the samples appear to change due to the inclusion of collagen,  
511 most significantly demonstrating the shift of the burst release behaviour in PLA-LEVO to  
512 sustained release behaviour within PLA-collagen-LEVO samples. This study is important in  
513 demonstrating that the modification of electrospun scaffolds can be achieved by  
514 incorporating natural polymers, such as collagen – and this modification may be useful in  
515 the compatibility and utilisation of tissue engineered structures within the human body.

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- 633



634 **Table & Figure Captions**

635 **Table 1:** Descriptive statistics of the electrospun fibre diameters.

636 **Table 2:** Multiple Comparisons.

637 **Figure 1:** The  $^1\text{H}$  NMR spectrum of polylactic acid in  $\text{CDCl}_3:\text{DMF}$ , 95:5, V/V%.

638 **Figure 2:** Histograms of fibre diameters (a) PLA Unloaded, (b) PLA-IRG, (c) PLA-LEVO, (d) PLA-  
639 Collagen (e) PLA-Collagen-IRG, and (f) PLA-Collagen-LEVO.

640 **Figure 3:** SEM Images of: (a) PLA Unloaded, (b) PLA-IRG, (c) PLA-LEVO, (d) PLA-Collagen, (e)  
641 PLA-Collagen-IRG, and (f) PLA-Collagen-LEVO.

642 **Figure 4:** AFM Images of (a) PLA Unloaded, (b) PLA-IRG, (c) PLA-LEVO, (d) PLA-Collagen (e)  
643 PLA-Collagen-IRG, and (f) PLA-Collagen-LEVO.

644 **Figure 5:** DSC Curves of (a) PLA-drug, (b) PLA-Collagen-drugs and (c) raw drugs Irgasan and  
645 Levofloxacin

646 **Figure 6:** XRPD Graphs of (a) PLA IRG Blends and (b) PLA LEVO Blends.

647 **Figure 7:** CAG Data for PLA-Drug and PLA-Collagen-Drug blends.

648 **Figure 8:** Drug release profiles of PLA-drug and PLA-collagen-drug scaffolds.

649 **Figure 9:** Images of antibacterial growth after 48 h. (a) PLA-IRG against E. Coli, (b) PLA-IRG  
650 against S. Aureus, (c) PLA-LEVO against E. Coli, (d) PLA-LEVO against S. Aureus, (e) PLA-  
651 Collagen-IRG against E. Coli, (f) PLA-Collagen-IRG against S. Aureus, (g) PLA-Collagen-LEVO  
652 against E. Coli, and (h) PLA-Collagen-LEVO against S. Aureus.

653 **Figure 10:** ToF-SIMS data of (a) PLA-IRG, (b) PLA-LEVO, (c) PLA-Collagen-IRG, and (d) PLA-  
654 Collagen-LEVO

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