

1 3D printed microneedle patches using
2 stereolithography (SLA) for intradermal insulin
3 delivery

4 *AUTHOR NAMES*

5 *Sophia N. Economidou¹, Cristiane Patricia Pissinato Pere¹, Andrew Reid², Md. Jasim Uddin³,*
6 *James F.C. Windmill², Dimitrios A. Lamprou^{4*}, Dennis Douroumis^{1*}*

7 *AUTHOR ADDRESS*

8 ¹Medway School of Pharmacy, University of Kent, Medway Campus, Central Avenue, Chatham
9 Maritime, Chatham, Kent ME4 4TB, United Kingdom

10 ²Centre for Ultrasonic Engineering, Department of Electronic and Electrical Engineering,
11 University of Strathclyde, 204 George St, Glasgow, G1 1XW, Scotland, United Kingdom

12 ³Department of Pharmacy, BRAC University, Bangladesh. Address: 41 Pacific Tower,
13 Mohakhali, Dhaka-1212, Bangladesh.

14 ⁴ School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, United
15 Kingdom

16 *Keywords*

17 3D printing, microneedles, inkjet coating, insulin, μ CT

18

19 Abstract

20 **3D printed microneedle arrays were fabricated using a biocompatible resin through**
21 **stereolithography (SLA) for transdermal insulin delivery. Microneedles were built by**
22 **polymerising consecutive layers of a photopolymer resin. Thin layers of insulin and sugar**
23 **alcohol or disaccharide carriers were formed on the needle surface by inkjet printing. The**
24 **optimization of the printing process resulted in superior skin penetration capacity of the**
25 **3D printed microneedles compared to metal arrays with minimum applied forces varying**
26 **within the range of 2 to 5N. Micro-CT analysis showed strong adhesion of the coated films**
27 **on the microneedle surface even after penetration to the skin. *In vivo* animal trials revealed**
28 **fast insulin action with excellent hypoglycaemia control and lower glucose levels achieved**
29 **within 60 min, combined with steady state plasma glucose over 4 h compared to**
30 **subcutaneous injections.**

31

32 Introduction

33 Transdermal Drug Delivery (TDD), the ability to effectively convey drugs through the human
34 skin, is an appealing concept, aiming at surpassing the pitfalls of the traditional administration
35 routes. The broader adoption of the transdermal route, however, is hampered by restrictions that
36 stem from the nature of the skin barrier itself, especially by the stratum corneum. Microneedles
37 (MNs) are small devices that can pierce this outermost, most impermeable layer of the human
38 skin and successfully deliver active substances such as drugs, Ribonucleic acid (RNA),
39 Deoxyribonucleic acid (DNA), and vaccines straight into the dermal microcirculation [1–4] .
40 Due to their small size they leave skin nerves intact upon insertion [5], while they increase

41 bioavailability since the drug does not pass through any metabolic systems [6]. The MN-
42 mediated drug delivery is realised through multiple strategies that employ solid, coated, hollow,
43 hydrogel-forming and soluble MNs [7].

44 Since their introduction over 20 years ago, MN systems have attracted significant attention for
45 their potential to replace traditional drug administration routes. In the field of diabetes type 1 and
46 some types of type 2, the vast majority of patients rely on subcutaneous needle injections for
47 insulin replacement, a treatment approach highly associated with reduced patient-compliance [8].
48 Pain, skin thickening due to recurring injections, needle phobia and insulin leakages on the skin
49 surface [9,10] have motivated vigorous research on MN-based systems for transdermal insulin
50 delivery.

51 Recent advances encompass the use of moulding techniques for the development of insulin-
52 loaded dissolvable MN systems. Wang *et al.* introduced a bioinspired MN system consisting of
53 dissolvable cross-linked poly(vinyl alcohol) (PVA) gel, catalase and glucose oxidase (GOx) that
54 responds to high glucose conditions by releasing insulin to the circulation. *In vivo* tests showed
55 that the systems were effective in maintaining normal blood glucose levels [11]. In another
56 study, modified alginate and hyaluronate were combined into a dissolvable, insulin-
57 encapsulating MN system. The MN arrays demonstrated good mechanical properties and skin
58 penetration capability while clinical studies demonstrated that the MNs almost fully dissolved
59 into the skin. The released insulin achieved a sustained hypoglycaemic effect and good relative
60 bioavailability of insulin, compared with subcutaneous injections [12]. Similar results in terms of
61 mechanical properties, relative insulin bioavailability and pharmacological activity were also
62 obtained by a study that manufactured composite dissolvable MNs [13]. The forenamed studies

63 demonstrate that MN can be a promising alternative to subcutaneous injections for insulin
64 therapy.

65 Nonetheless, there are several hampering parameters that need to be taken under consideration
66 for the development of transdermal insulin delivery systems. The first relates to the time needed
67 for the detection of the drug into the systemic circulation; dissolvable systems are highly
68 dependent on the dissolution rate of the materials encapsulating the drug and may not be suitable
69 for fast insulin administration. To circumvent that issue, Ross *et al.* developed a solid MNs-
70 based system, coated with insulin-containing formulations through inkjet printing [14]. The use
71 of this technology permitted the accurate deposition of uniform and homogeneous coatings, with
72 high reproducibility. The implementation of inkjet printing for the development of thin layers on
73 the microneedle surface resulted in rapid insulin release within the first 20 min.

74 Another pitfall stems from the use of moulding techniques, that involve a series of multiple,
75 often time-consuming steps. It is evident, that the upscaling of such processes can be
76 challenging. Furthermore, there is a lack of clinical data related to cytotoxicity of materials used
77 for moulded microneedles which actually limits their applications. Another important
78 disadvantage is the limited drug loading in polymeric microneedles without affecting their
79 mechanical properties and piercing capacity. In order to circumvent this issue several authors
80 proposed the use of large patches which in turn results in difficulties to apply the arrays in a
81 uniform manner and subsequently in dose variation of the administered substances. Recently,
82 efforts have been made on the integration of the revolutionary technology of 3D printing as a
83 manufacturing method for MN-based systems. 3D printing or Additive Manufacturing (AM) is a
84 family of technologies that implement layer-by-layer processes to fabricate physical models,
85 based on a Computer Aided Design (CAD) model. 3D printing permits the fabrication of high

86 degrees of complexity with great reproducibility, in a fast and cost-effective fashion [15–18]. In
87 the field of transdermal drug delivery systems, the use of photopolymerization-based techniques
88 such as Stereolithography (SLA), Digital Light Processing (DLP) and Two-Photon-
89 Polymerization (2PP) for the development of MNs has been reported [19–22]. Gittard *et al.*
90 fabricated MNs of various geometries for wound healing applications using a DLP system. The
91 MNs were then coated with silver and zinc oxide thin films by pulse laser deposition and their
92 antimicrobial character was verified [23]. In another study, drug-loaded MNs were developed
93 when a skin anticancer drug was incorporated into the photo-sensitive polymer blend prior to
94 photopolymerization through a micro-stereolithographic (DLP) apparatus [24].

95 In this study, 3D printed MN arrays featuring two different MN designs, pyramid and spear,
96 were developed employing a commercial SLA printer and a biocompatible Class 1 polymer. The
97 3D printed arrays were subsequently coated with insulin–sugar films using inkjet printing.
98 Mannitol, trehalose and xylitol were used as insulin carriers to preserve insulin activity prior to
99 the deposition of active films on the MNs surface. *In vitro* and *in vivo* studies demonstrated rapid
100 insulin release from the coated MN systems. The usage of SLA for 3D printing of microneedle
101 arrays is anticipated to overcome the existing disadvantages of conventional techniques by
102 providing high precision, rapid fabrication, reduced processing steps and freedom to print a wide
103 range of shapes.

104 Materials and methods

105 Materials

106 The insulin employed in this study was bovine and was procured in a 10 mg mL⁻¹ solution from
107 Sigma-Aldrich (Gillingham, UK). Xylitol (Xylisorb® 90) and mannitol (Pearlitol®) were

108 donated by Roquette Freres (France) while trehalose dihydrate was bought from Sigma-Aldrich
109 (Gillingham, UK). The resin used to fabricate the MNs was the biocompatible Class I resin,
110 Dental SG, by Formlabs. Streptozocin ($\geq 75\%$ α -anomer basis, $\geq 98\%$) and citric acid were both
111 purchased from Merck Chemical Co. (Darmstadt, Germany). All solvents were of analytical
112 grades.

113

114 Additive manufacturing of microneedles

115 The MN arrays were designed using an engineering software (SolidWorks, Dassault Systems) as
116 patches of 15x15x1 mm. The patches featured two different needle shapes, a pyramid and a flat
117 spear shaped that geometrically resembled the shape of metallic MNs that has been studied
118 elsewhere [16]. This design, named ‘spear’ in the framework of this study, had base dimensions
119 of 0.08x1 mm, while the dimensions of the base for the pyramid MN were 1x1 mm. The length
120 of all MNs was 1 mm and all patches had a 6x8 needle layout, yielding 48 MNs per patch. The
121 arrays were 3D printed using the Form 2 SLA printer by Formlabs with high resolution
122 capabilities (25 and 140 microns for z and x axes, respectively). After fabrication, the arrays
123 were washed in isopropyl alcohol bath to remove unpolymerized resin residues and then cured
124 for 60 min at 40 °C under UV radiation using the MeccatroniCore BB Cure Dental station.

125 Coating of microneedles through inkjet printing

126 An inkjet printer was employed (NanoPlotter II, Gesim, Germany) to print thin insulin-sugar
127 films on the surface of the 3D printed MNs. The inkjet printer forms the drug-containing films
128 depositing multiple layers of insulin – sugar droplets on each microneedle using a piezo-driven
129 dispenser (PicPip 300). In each coating cycle (layer), the dispenser jetted 2 droplets of

130 formulation in 10 spots along each needle's longitudinal axis. A total of 92 coating cycles
131 resulted in a 10 UI (350 μ g) of insulin per array. The coated arrays were then incubated at room
132 temperature for 24 hours to allow the evaporation of the solvent (de-ionised water) and the
133 formation of uniform films. For the purposes of this study three coating formulations were used,
134 consisting of insulin:xylitol (5:1 wt/wt), insulin: mannitol (5:1 wt/wt) and insulin:trehalose (5:1
135 wt/wt) as 2% solid content. Prior to the coating process, the arrays were mounted on a metal stub
136 at 45° relative to the dispenser, while its tip (50 μ m) was placed close to the MN surface to avoid
137 losses of material.

138 Scanning electron microscopy (SEM)

139 The coated MN arrays were mounted onto aluminium stubs using a double-sided carbon
140 adhesive tape (Agar Scientific, UK). Each coated MN array was examined by SEM (Hitachi SU
141 8030, Japan) using a low accelerating voltage (1.0kV). A low accelerating voltage was used to
142 avoid electrical charges on the MNs. The images of coated MNs were captured digitally from a
143 fixed working distance (11.6 mm) using different magnifications (e.g. 30, 80, 110 or 120 x).

144 X-Ray Computer Micro Tomography

145 X-Ray Micro Computer Tomography (μ CT) scans were performed on coated 3D printed
146 pyramid MN. The equipment employed was a Bruker Skyscan 1172, with an SHT 11 Megapixel
147 camera and a Hamamatsu 80kV (100 μ A) source. The samples comprised of 3D printed pyramid
148 MN coated with the three insulin/sugar formulations; Sample A: insulin:xylitol (5:1 wt/wt),
149 Sample B: insulin: mannitol (5:1 wt/wt) and Sample C: insulin:trehalose (5:1 wt/wt). After the
150 scans of the coated arrays were performed, the arrays were inserted in 8-ply strips of parafilm,
151 applying a force of 5 N, to examine the performance of the coating during piercing and to

152 investigate whether any coating material will remain on the parafilm surface, causing drug
153 losses. Moreover, the penetration depth was measured. The samples were mounted vertically on
154 a portion of dental wax and positioned 259.4 mm from the source. No filter was applied to the X-
155 Ray source and a voltage of 80 kV was applied for an exposure time of 1,050 ms. The images
156 generated were 2,664 x 4,000 pixels with a resolution of 6.75 μm per pixel.

157 A total of 962 images were taken in 0.2° steps around one hemisphere of the sample with the
158 average of 4 frames taken at each rotation step. The images were collected and a volumetric
159 reconstruction of the sample generated by Bruker's *CTvol* software. The threshold for this
160 attenuation signal was set manually to eliminate speckle around the sample, and then further
161 cleaned with a thresholding mask using Bruker's *CTAn* software. The images produced by the
162 μCT are based on the level of attenuation through the sample, which is dependent on the thickness
163 of the material and its absorption coefficient. Here, it is assumed that the absorption coefficient is
164 linearly proportional to the density of the material and the resulting densities expressed in
165 Hounsfield Units (HU), with -1000 being the density of air and 0 being the density of water.

166 Circular Dichroism (CD)

167 Insulin solution and the respective solutions of the insulin-sugar films were diluted to 1.0 mg
168 mL^{-1} in deionised water and the spectra were recorded at 20 $^\circ\text{C}$ between 190 and 260 nm by CD
169 (Chirascan, Applied Photophysics, UK) using a 0.1 mm polarization certified quartz cell
170 (Hellma). Spectra were recorded using a step size of 1 nm, a bandwidth of 1 nm and an
171 acquisition time of 1 sec. Four scans were recorded for each sample, averaged and a
172 corresponding spectrum of water was subtracted from each spectrum. For estimation of the

173 secondary structural composition of insulin, the CD spectra were evaluated using the CD SSTR
174 method [25].

175 Raman Spectroscopy

176 The films and their respective components were analysed using Raman microscopy (Jobin Yvon
177 LabRam I) with a laser of 532 nm wavelength coupled with an optical microscope with 50x
178 objective.

179 Penetration studies through porcine skin

180 The effect of the MN geometry on the force required to pierce the skin has been documented
181 [26]. In this study, to determine the effect of needle shape on the force required for skin
182 penetration, piercing tests using porcine skin were conducted. Identical piercing tests were
183 carried out using metallic MN arrays that have been studied and are described in literature [16],
184 to maintain a frame of reference with the respective studies. A texture analyser was employed,
185 and the MN array was mounted on the moving probe using double-sided adhesive tape. Prior to
186 testing, the porcine skin samples were placed in waxed petri dishes. Continuous force and
187 displacement measurements were recorded to identify the point of needle insertion. The speed of
188 the moving probe was 0.01 mm s^{-1} .

189 Axial force mechanical testing of MNs

190 To evaluate the mechanical behaviour of the 3D printed microneedles, fracture testing under
191 axial loading was performed. The arrays were fixed onto a metal plate and were pressed against a
192 flat metal block attached to the moving head of a Tinius Olsen testing machine, until a pre-set
193 displacement of $500 \text{ } \mu\text{m}$ (height/2) was reached. Continuous force and displacement

194 measurements were recorded to identify the point of needle failure. The speed of the moving
195 probe was 1 mm/s and the experiments were replicated 5 times for each design.

196 Preparation of porcine skin for in vitro release of insulin

197 The release of insulin from the coated MNs through abdominal porcine skin was studied using
198 Franz diffusion cells (PermeGear, Inc., PA, USA). The full thickness abdominal porcine skin
199 was collected from a local slaughterhouse (Forge Farm Ltd, Kent, UK) and was then shaved
200 using a razor blade. The fatty tissue below the abdominal area of porcine skin was removed with
201 scalpel and then pinned onto polystyrene block and wiped with 70 % ethanol. The skin was then
202 cut by applying the dermatome at an angle of $\pm 45^\circ$ (Padgett dermatome, Integra LifeTMSciences
203 Corporation USA). The thickness of the skin was measured by using a calliper and the tissue
204 disks of the required dimensions were cut for the Franz diffusion cells using a scalper. The skin
205 tissue (1.0 ± 0.1 mm thick) was placed onto filter paper soaked in a small amount of saline
206 phosphate buffer (pH 7.4) for 2 h.

207 In vitro release of insulin through porcine skin

208 A total diffusion area of 1.1 cm^2 was used to assess the insulin release. The MN arrays were
209 inserted into the abdominal porcine skin samples for 30 s, via manual finger pressure. The
210 sample was then mounted onto the donor compartment of a Franz diffusion cell. The temperature
211 of the Franz cells was maintained at 37°C using an automated water bath (Thermo Fisher
212 Scientific, Newington, USA). Sample fractions ($6\text{-}6.5 \text{ mL h}^{-1}$) were collected using an auto-
213 sampler (FC 204 fraction collector, Gilson, USA) attached to the Franz diffusion cells system.
214 Statistical analysis for the drug release was performed by using a Mann-Whitney nonparametric

215 test and t-test analysis for the in vivo studies (InStat, GraphPad Software Inc., San Diego, CA,
216 USA), where samples were considered as statistically significant at $p < 0.05$.

217 High-Performance Liquid Chromatography (HPLC)

218 The amount of insulin collected from the receptor fluid was determined by HPLC (Agilent
219 Technologies, 1200 series, Cheshire, UK) equipped with a Phenomenex Jupiter 5u c18 300 Å,
220 LC Column (250×4.60 mm, particle size 5 μm, Macclesfield, UK). The mobile phase consisted
221 of water with 0.1 % Trifluoroacetic Acid (TFA) and acetonitrile with 0.1% TFA (66:34v/v), with
222 a 1 mL min⁻¹ flow rate. The column was equilibrated at 35°C, the injection volume was 20 μL
223 and the eluent was analysed with a UV detector at 214 nm. The results were integrated using
224 Chemstation® software and the samples analysed in triplicates.

225 In vivo release in diabetic mice

226 Prior to the induction of diabetes, Swiss albino female mice (120 ± 10 g) were allowed free
227 access to solid bottom cages with controlled diet and water for 3 days. Mice were subcutaneously
228 injected on the flank with streptozotocin (70 mg kg⁻¹) in citric acid buffer (pH 4.5) to produce a
229 diabetic animal model. To confirm the induction of diabetes, the fasting blood glucose level was
230 measured at scheduled times using a one-touch glucometer (ACCU-CheckVR Active, Roche,
231 Germany). After one week, mice with blood glucose exceeding 300 mg/dl were considered as
232 diabetic. The diabetic animals were anaesthetised and shaved carefully using an electric razor
233 (Panasonic, USA) 24 hours prior to the experiments. Furthermore, the diabetic mice were fasted
234 for 12 hours before the beginning of the study, receiving only water and libitum. The mice were
235 randomly divided into three groups (n=3 for each group): (1) untreated group as negative control;
236 (2) subcutaneous injection (SC; 0.2 IU/animal) as positive control; (3) 3D printed MN (0.2

237 IU/array). The 3D printed MN arrays were applied onto the dorsal skin of the animals using
238 adhesive tape (3M, USA) to prevent any dislodgement during therapy. After 2 hours, the 3D
239 printed MN patches were removed. For all groups, blood samples were collected from the
240 jugular vein at 0, 1, 2, 3 and 4 hours after the insulin administration and the blood glucose level
241 was measured using the glucometer mentioned. Plasma insulin concentrations were measured via
242 an insulin-EIA Test kit (Arbor Assays, MI, USA). The treatment strategy is described in Table 1.

243 All animal experiments throughout this study were approved by the Research Ethics Committee
244 (reference number 0003/17, Department of Pharmacy, Southern University Bangladesh) and
245 conducted according to the Southern University Bangladesh policy for the protection of
246 Vertebrate Animals used for Experimental and Other Scientific Purposes, with implementation
247 of the principle of the 3Rs (replacement, reduction, refinement). No skin reactions to MNs
248 occurred.

249 Pharmacodynamic and pharmacokinetic profile of insulin-coated 3D printed MNs

250 The minimum glucose level (C_{min}) and the time point of minimum glucose level (T_{min}) were
251 calculated from the plasma glucose level versus time curve. The relative pharmacological
252 availability (RPA) was calculated using equation 1.

$$253 \text{ RPA(\%)} = (\text{AAC}_{3\text{DMN}} \times \text{doscsc}) / (\text{AAC}_{\text{sc}} \times \text{dose}_{3\text{DMN}}) \times 100 \quad (\text{Eq.1})$$

254 Where $\text{AAC}_{3\text{DMN}}$ indicates the area above the curve after the application of the insulin-coated
255 3D printed MNs, and AAC_{sc} shows the area above the curve after the subcutaneous injection of
256 insulin.

257 The maximum plasma insulin concentration (C_{max}) and the time point of maximum plasma
258 insulin concentration (T_{max}) were calculated from the plasma insulin concentration ($\mu\text{IU/ml}$)
259 versus time curve. The relative bioavailability (RBA) was determined using equation 2.

$$260 \text{ RBA}(\%) = (\text{AUC}_{3\text{DMN}} \times \text{doses}_{\text{sc}}) / (\text{AUC}_{\text{sc}} \times \text{dose}_{3\text{DMN}}) \times 100 \quad (\text{Eq.2})$$

261 Where $\text{AUC}_{3\text{DMN}}$ indicates the area under the curve after the application of the insulin-coated
262 3D printed MNs, and AUC_{sc} shows the area under the curve after the subcutaneous injection of
263 insulin.

264 Results and Discussion

265 Additive Manufacturing and printability of microneedles

266 MN arrays featuring pyramid and spear needles were 3D printed using a commercial SLA printer
267 based on digital CAD designs developed via appropriate engineering software. The polymer
268 employed was a photo-sensitive Class I resin which has been FDA approved. All arrays were
269 washed and subsequently cured under UV radiation in a controlled temperature environment to
270 improve the material's mechanical performance.

271 The capability of 3D printing technology to manufacture complex structures reproducibly and
272 accurately in a one-step-fashion, was exploited in this work to build different designs of MN
273 arrays. Although the degree of complexity that can be achieved through 3D printing is often not
274 achievable through many conventional techniques of MN manufacturing, the technology is
275 hampered by restrictions in terms of resolution that can affect the formation of sharp MN tips.
276 Conventional low-budget SLA printers have a maximum resolution of 100 microns that is
277 governed by the size of the laser focal point and restricts the minimum size of MN tip that can be

278 formed. The MNs designed in the framework of this study featured a tip of 100 microns by
279 design and their penetration capability through porcine skin was tested to verify that they will
280 successfully and painlessly pierce the skin.

281 The printability of MNs was further improved when printing-in-an-angle was implemented,
282 leading to finer, sharper MN tips. An innate characteristic of the SLA technology is the
283 interdependence between the print quality and the cross-sectional area in the z-axis; the smaller
284 the z-axis cross-sectional area, the better the quality. This stems from the peel-off function of the
285 printing process, according to which, after the completion of each layer, a wiper slides and peels
286 the structure off the bottom of the resin tank. Larger z-axis cross sectional areas lead to greater
287 forces applied by the wiper, which can deform the printed structures. Orienting the part to
288 minimise the contact area of the structure to the resin tank avoids the possible distortions during
289 the peel-off process and leads to better print quality.

290 SLA parts are considered mechanically isotropic which ensures the mechanical properties of the
291 arrays are not affected by the angle of printing. In addition, an influential factor of the
292 mechanical performance of the MN arrays is the selection of post-process curing parameters
293 (time and temperature). Further research is required to determine the effect of those factors on
294 the overall mechanical and piercing behaviour of the MN arrays. In this work, the MNs were
295 cured in a UV chamber for 60 min in temperature of 40 °C. It is demonstrated in this study that
296 those parameters yielded systems that successfully pierced through porcine skin requiring small
297 forces, with no needle failure occurring.

298 As discussed above due to technical limitations of the existing MN manufacturing techniques
299 (e.g. moulding, lithography) such as limited drug loading, dose consistency and scalability issues

300 there are no commercialized products. Polymeric MNs are fabricated using moulding approaches
301 while metal MNs implement dip – coating techniques which renders both approaches impractical
302 for large scale manufacturing. In contrast scale-up of SLA printed MNs is directly related to the
303 usage of large volume printers or the in-line arrangement of existing printers. We envisage that
304 the implementation of SLA printed MNs will open new horizons for transdermal drug delivery
305 due to the low cost of the printers, printing inks and fast fabrication times.

306 SEM analysis demonstrated that through the use of the SLA technology, uniform and
307 reproducible arrays were developed (Fig. 1a,b). In Fig. 1, the high consistency and
308 reproducibility of the MN layers is depicted, and the formation of sharp tips is demonstrated. It is
309 evident, that the high-resolution capabilities of the printer allowed the parallel fabrication of
310 identical and reproducible arrays with characteristics that favour the skin insertion.

311 Coating of microneedles through inkjet printing

312 Insulin and sugar alcohol coatings were formed on the surface of the 3D printed MNs using
313 inkjet printing and a piezoelectric dispenser. A similar process was developed in earlier studies
314 for coating metallic MNs [16,27], where the applied voltage (mV) and pulse duration (ms) were
315 tuned to achieve the production of droplets of 300 pL volume with particle size of 100-110 μm .
316 Fig. 1c,d illustrates the uniformity and reproducibility of the coatings on the MN surface without
317 any losses of material in the form of satellite droplets on the substrate. Moreover, it is
318 demonstrated that the consecutive jetting cycles produced drug-containing films that are smooth
319 and level in comparison to other techniques such as dip coating that may yield voluminous and
320 inconsistent coatings. This smooth morphology of the films prevents the losses of drug during
321 MN insertion that occur when bulky coatings remain on the skin surface.

322 The drug carriers selected were two alcohol sugars (xylitol, mannitol) and a disaccharide
323 (trehalose). Those excipients have been reported to favour the immediate coating dissolution in
324 the skin and to enhance insulin stability in solid state [28–30] .

325 X-Ray Computer Micro Tomography

326 The coated 3D printed pyramid MN arrays were scanned using the Bruker Skyscan 1172 and an
327 overview of the array is presented in Fig. 2a. For sample A, an average needle base area of 1.095
328 mm² and an average needle height of 1.034 mm were measured. For samples B and C, the
329 average needle base areas were measured as 1.065 mm² and 1.091 mm² and the average needle
330 heights as 1.040 mm and 1.038 mm, respectively. The average interspacing of the pyramids
331 between the centre points was 1.842 mm, 1.865 mm, and 1.864 mm between columns, and 1.788
332 mm, 1.810 mm, and 1.796 mm between rows for samples A, B and C respectively. Scans taken
333 from the left-hand side of the arrays illustrate the thin coating films fabricated through inkjet
334 printing, in comparison with respective ones taken from the back side of the array (Fig. 2b,c).

335 The relative density of the MNs relative to the control sample (uncoated 3D printed pyramid
336 MNs) showed an increase of approximately 200 HU between all coated samples and the control
337 (Fig. 3a,b). Profile lines across a row of MNs revealed a coffee-ring effect in the density of the
338 coating material deposition. While denser material was distributed randomly within each of the
339 MNs, a fringe layer of 10-15 µm was apparent with the effect being most pronounced in the
340 insulin:xylitol coated sample (Fig. 3c).

341 Penetration experiments in 8-ply strips of parafilm were performed applying a 5 N force (Fig. 4).

342 The penetration depth was measured as 559 µm, 662 µm and 650 µm for samples A, B and C,

343 respectively. The μ CT scans illustrate that the coating stays on the MN surface throughout the
344 piercing process and there is no material remaining on the parafilm surface.

345 Circular Dichroism

346 Circular dichroism (CD) spectroscopy is a reliable technique for the evaluation of the secondary
347 structure of proteins in a solution. The influence of the two polyols and the disaccharide on
348 insulin molecule as well as their interactions were studied using CD and the estimation of insulin
349 secondary structure was performed by CDSSTR method [31,32].

350 The far-UV CD spectra of insulin and insulin-sugar films (Fig. 5a) were found to be coincident
351 with the one of standard insulin solution, showing double minima around 210 and 222 nm which
352 are typical of predominant α -helix structure proteins as already reported elsewhere [28,33–35].
353 However, a slight decrease in Molar ellipticity is noted when insulin solution is dried which is
354 also supported by the decrease of the estimated percentage of α -helix and increase of the β -sheet
355 content. Such behaviour may be indicative of the unfolding tendency of insulin during
356 dehydration [36]. Interestingly, once the sugars were added, all the respective insulin-sugar films
357 spectra showed higher Molar intensities than the insulin film alone, indicating an increase in the
358 α -helix content. The protective property of those sugars can be explained by the water
359 replacement mechanism which proposes that sugars may maintain the three-dimensional
360 structure of proteins by hydrogen-bonding with them [37,38]

361 Among the tested sugars, xylitol presented the best capability to maintain insulin in its native
362 secondary structure with even higher amounts of α -helix content. The reason for this still remains
363 unclear and further research is needed.

364 Raman Spectroscopy

365 In this work, the Raman spectrum of native insulin shows a strong peak at 1661 cm^{-1} due to the
366 amide I mode of α -helix structure and a shoulder at 1682 cm^{-1} which is attributed to random coil
367 form as previously reported by Yu et al. [40]. Distinctive peaks of sugars were not found in
368 Raman mainly because insulin was 5 times more concentrated than the sugars in the films.
369 Likewise, the amorphous nature of the dried formulation (XRD analysis - data not shown) is
370 unlikely to afford a strong Raman signal.

371 Overall, insulin-sugar formulations showed similar Raman spectra to the native insulin (Fig. 5b).
372 Nonetheless, a slight shift in amide I band position can be seen for all formulations. Amide I
373 band of insulin-xylitol and insulin-mannitol formulations was shifted towards greater frequency,
374 1663 cm^{-1} and 1662 cm^{-1} , respectively, while insulin-trehalose band was shifted to lower
375 frequency at 1658 cm^{-1} . Those events were also reported by Carpenter and Crowe [37] and
376 Souillac et al. [41], who described that those changes might be due to the different effect of each
377 sugar on the vibrational spectra of insulin as well on the hydrogen bonding and couplings
378 between the adjacent peptide units.

379 Many researchers have investigated the protective properties of different sugars on polypeptides,
380 proteins and biomolecules [42–44]. It has been advocated that protein aggregation and
381 denaturation can be prevented by using carbohydrates as protectants. Protein protection by the
382 sugars in a dried system can be explained by the water replacement mechanism which suggest
383 the sugars may substitute water molecules around the biomolecules of proteins, maintaining its
384 three-dimensional structure by providing sites with hydrogen-bonding species [37,45,46].

385 Zeng *et al.* studied the impact of relative humidity (RH) on dehydration of insulin crystals and
386 they found the hydration water from insulin crystal can be gradually excluded when the RH is

387 decreased. They used the high frequency region in Raman spectroscopy to access the band at
388 $\sim 3450\text{cm}^{-1}$ which is caused by both water and amino acid residues with O-H groups. A
389 continuous dropping of the O-H stretching band around 3450 cm^{-1} was observed while the RH
390 was reduced, indicating dehydration of the molecule [47].

391 From Fig. 5b it can be seen that for all formulation the S-S vibration bands are located close to
392 513 cm^{-1} suggesting that all disulphide bonds are in an adopted more stable gauche – gauche -
393 gauche conformation [47,48] as a result of the complete water removal during inkjet printing.
394 Tyr residues present Raman peaks at 642, 828, 852, and 1174 cm^{-1} , while the 1206 cm^{-1} peak is
395 related to both Tyr and Phe residues. Furthermore, the ratios of $I_{852/1828}$ and $I_{1174/11206}$ varied from
396 $0.91 - 1.02$ and $0.77 - 0.81$ respectively. These values are much lower compared to those
397 observed from Zeng et al. [47], for insulin crystals at very low RH (2%). This phenomenon
398 suggests significant water loss of the coated formulation and stronger H – bonding interactions.

399 Vibrational modes in the area of $1100 - 1300\text{ cm}^{-1}$ have shown to be sensitive to the changes of
400 hydrogen bonds which involve the phenolic hydroxyl groups of Tyr residues and particularly the
401 ν_{7a} frequency. In Fig. 5b the ν_{7a} has a frequency of 1275cm^{-1} which is a robust evidence that the
402 phenolic OH group of Tyr is strongly hydrogen bonded to a base atom [49].

403 Penetration studies through porcine skin

404 The 3D printed pyramid and spear shaped MN arrays were tested for their porcine skin
405 penetration capability using a texture analyser. Identical experiments were performed using
406 metallic MNs and the results were compared to the respective ones obtained from the 3D printed
407 MN experiments. All piercing tests were successful with no MN damage or failure. Throughout
408 each test, measurements of force and displacement were taken (Fig. 6a).

409 All curves presented an initial linear segment (displacement < 0.3 mm); after that, the slope was
410 changing constantly until a maximum force value was reached and a steep decrease of the force
411 was observed. This value is identified as the maximum force required for MN insertion [26]. The
412 non-linear behaviour of the force-displacement curve indicates that the process of MN insertion
413 to the skin is comprised of small penetrations where the MNs gradually tear the skin, before the
414 load reaches the maximum value that makes the insertion abrupt [50]. The maximum force
415 required for the MNs to successfully pierce the skin plays a crucial role when different MN
416 designs need to be compared. As presented in Fig. 6b, the pyramid MN required the least amount
417 of force to penetrate the porcine skin.

418 Axial force mechanical testing of MNs

419 The two studied MN designs were tested under compressive axial loading to determine the force
420 of microneedle fracture as a function of geometry. The force vs displacement measurements and
421 respective fracture strength values are presented in Fig. 7.

422 The two designs exhibited different mechanical behaviours during testing. For both designs, the
423 recorded force increased until the ultimate load was reached, and fracture occurred. For the spear
424 MNs, the point of fracture appears as a peak at approximately 175 N, followed by a drop of the
425 recorded load; as the MNs were kept being pressed against the metal block after fracture, the
426 load was considerably decreased. On the contrary, the pyramid geometry showed a discontinuity
427 at approximately 457 N which is identified as the point of initial needle failure. Afterwards, the
428 load kept increasing as the microneedles kept being compressed. This difference in mechanical
429 behaviour is attributed to the different modes of needle failure. On the one hand, the spear MNs
430 fractured in the lateral direction, perpendicular to the loading axis, a finding that was confirmed

431 by visual observation. This mode of failure was expected, due to the small thickness of the MNs
432 in that direction, which translates to minimisation of area, thus increased stress fields. On the
433 other hand, the pyramid MNs failed under pure compression, with the tip failing first and
434 additional, increasing force required for the compression of the remaining MN body. These
435 findings verify that both designs are safe for application since the fracture strengths of the arrays
436 are far greater than the respective forces needed for needle penetration through porcine skin.
437 They also confirm that the pyramid geometries present the best potential between the two studied
438 designs.

439 In vitro release of insulin through porcine skin

440 The in vitro insulin release studies from 3D printed pyramid and spear MNs were investigated
441 using porcine skin in Franz cells. The used carriers, mannitol, trehalose and xylitol not only
442 preserved insulin in its native form but also provided fast dissolution rates. As shown in Fig.
443 8a,b for the pyramid designs approximately 80% of insulin was released in the first 2 min with
444 86 – 92% within 8 min. The rapid release profiles were obtained for all insulin carriers and no
445 statistical difference was observed (two-tail $p = 0.0021$).

446 The coating of each pyramid side resulted to higher surface area exposed for hydration and thus
447 faster hydration rates. In contrast, the spear 3D printed designs presented slightly slower insulin
448 release rates with 62 – 70% and 81 – 84% released within 2 min and 8min respectively. Overall,
449 the rapid insulin release rates of the 3D printed MNs was attributed to the hydrophilic nature of
450 the three carriers and the thin coating layers (10 – 15 μ m) as shown from the μ CT analysis.

451 In vivo transdermal delivery of insulin in diabetic mice

452 Diabetes was successfully induced in mice after 7 days of streptozotocin administration. The
453 preliminary diabetes (hyperglycemia) was demonstrated as 340 ± 10 mg/dl. The diabetic mice
454 were divided into three groups: Untreated (negative control), subcutaneously (SC) injected
455 (positive control) and treated with the 3D printed insulin-coated MNs. Fig. 9 shows the
456 application process of the 3D printed MN arrays.

457 The dose 0.2 IU/array was selected in order to avoid hypoglycaemia in mice for 4 h. The
458 comparative studies on different delivery strategies in plasma glucose levels are shown in Fig.
459 10a. Insulin-coated 3D printed MN arrays showed a remarkable steady state hypoglycaemia
460 effect (32.8% from total value) in comparison to negative and positive control. After an hour,
461 subcutaneous injection (0.2 IU/injection) facilitated a rapid increase in insulin concentration in
462 blood and hence the decrease in plasma glucose level was approximately 30.1% from its primary
463 value. A comparable blood glucose regulation was observed between the SC group and the MN
464 group which was achieved within 1 h [51]. Interestingly, the 3D printed patch presented the same
465 rate to reach its lowest glucose level compared to the SC injection. In similar study
466 biodegradable MN patches T_{min} of glucose levels was achieved within 2 h with administrated
467 doses of 5 – 10 IU per patch [13]. The main reason for the faster glucose rates is that in *Zhang et*
468 *al.* (2018) insulin was encapsulated in moulded MN patches while for the 3D printed patches is
469 applied on the microneedle surface with very hydrophilic thin layers resulting in rapid insulin
470 release. Moreover, previous studies have shown that microneedle injection of insulin to human
471 diabetic subjects was chosen over hypodermic infusion and that pharmacokinetics were faster
472 when insulin was administered to the skin compared to the subcutaneous injections [52].

473 Although the plasma glucose level versus time profile was similar to previous findings [53–57]
474 the steady state plasma glucose level was maintained up to 4 h while the untreated group

475 (negative control) remained unchanged (no hypoglycaemia) for the same period. These findings
476 suggest that insulin is being released from the 3D printed MNs to the mice blood stream via
477 passive diffusion to blood capillary.

478 Fig. 10b illustrates the plasma insulin concentration versus time where both SC and 3D printed
479 MN groups achieved the highest amount of plasma insulin concentration after 1 h of
480 administration. The control group did not show any detectable plasma insulin concentration. The
481 highest insulin level of the 3D printed MNs is slightly lower to the SC injection but no statistical
482 difference was observed. As shown in Fig. 10b after post – administration for 4 h the serum
483 insulin of 3D printed microneedles was higher to the SC injection.

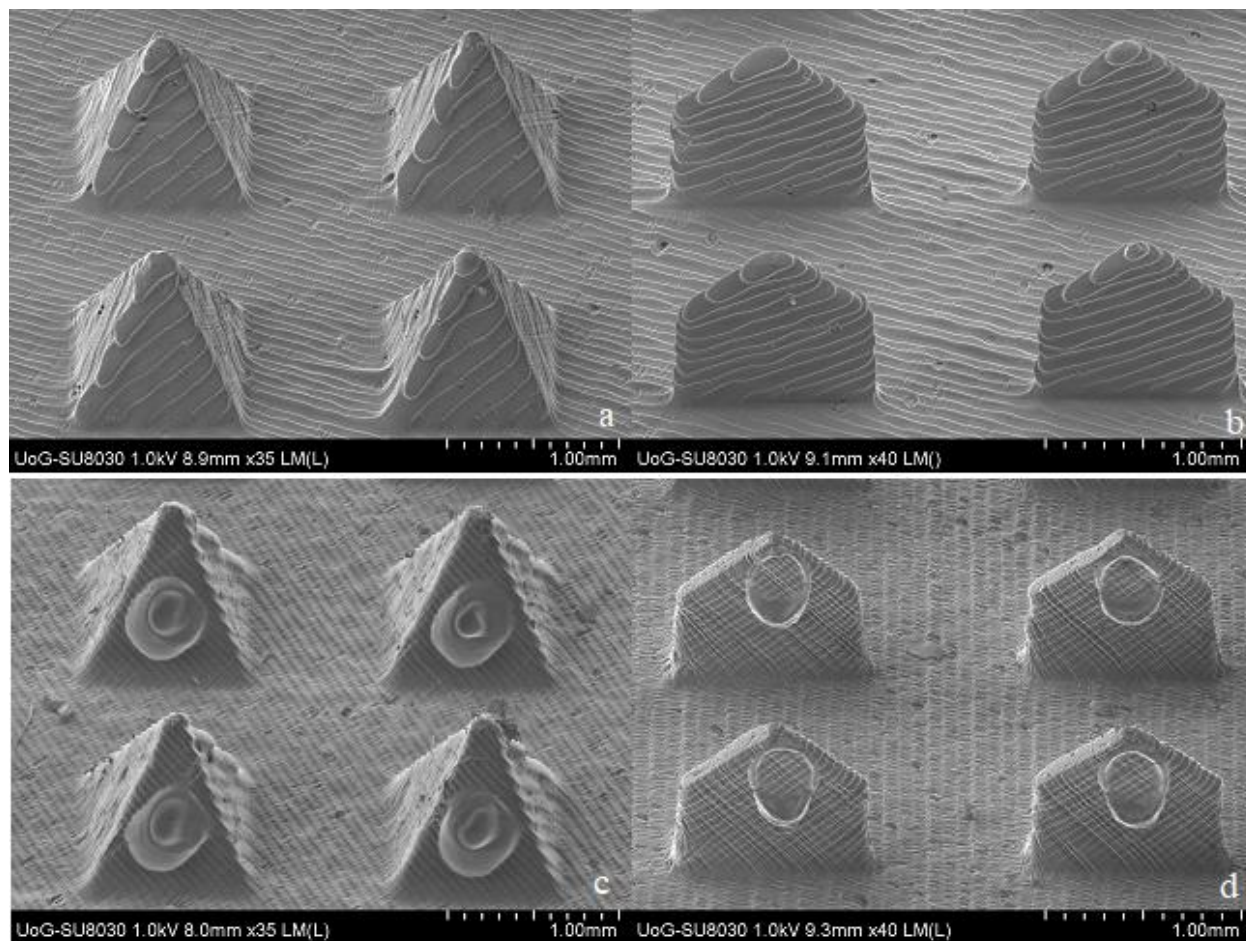
484 Tables 2 and 3 represent the pharmacodynamic parameters of plasma glucose levels and the
485 pharmacokinetic parameters for plasma insulin concentrations, respectively. The RPA and RBA
486 for the insulin-coated 3D printed MNs group were both about 85-96%. These results indicate that
487 insulin released from 3D printed MNs was almost completely absorbed from the skin into the
488 systemic circulation, and the pharmacological activity of the released insulin remained intact
489 after the delivery with the 3D printed MNs.

490 Conclusions

491 MN arrays of high quality and reproducibility, featuring spear and pyramid-shaped needle
492 geometries, were successfully fabricated by a biocompatible resin using stereolithography. The
493 3D printed polymeric MNs required low forces to penetrate porcine skin, in comparison with
494 metallic MNs. Uniform and accurate insulin-sugar thin layers were applied on the surface of the
495 MNs through inkjet printing, with no satellite droplets detected on the substrate. The insulin
496 integrity was found to be preserved by all carriers, namely the α -helix and β -Sheet, with xylitol

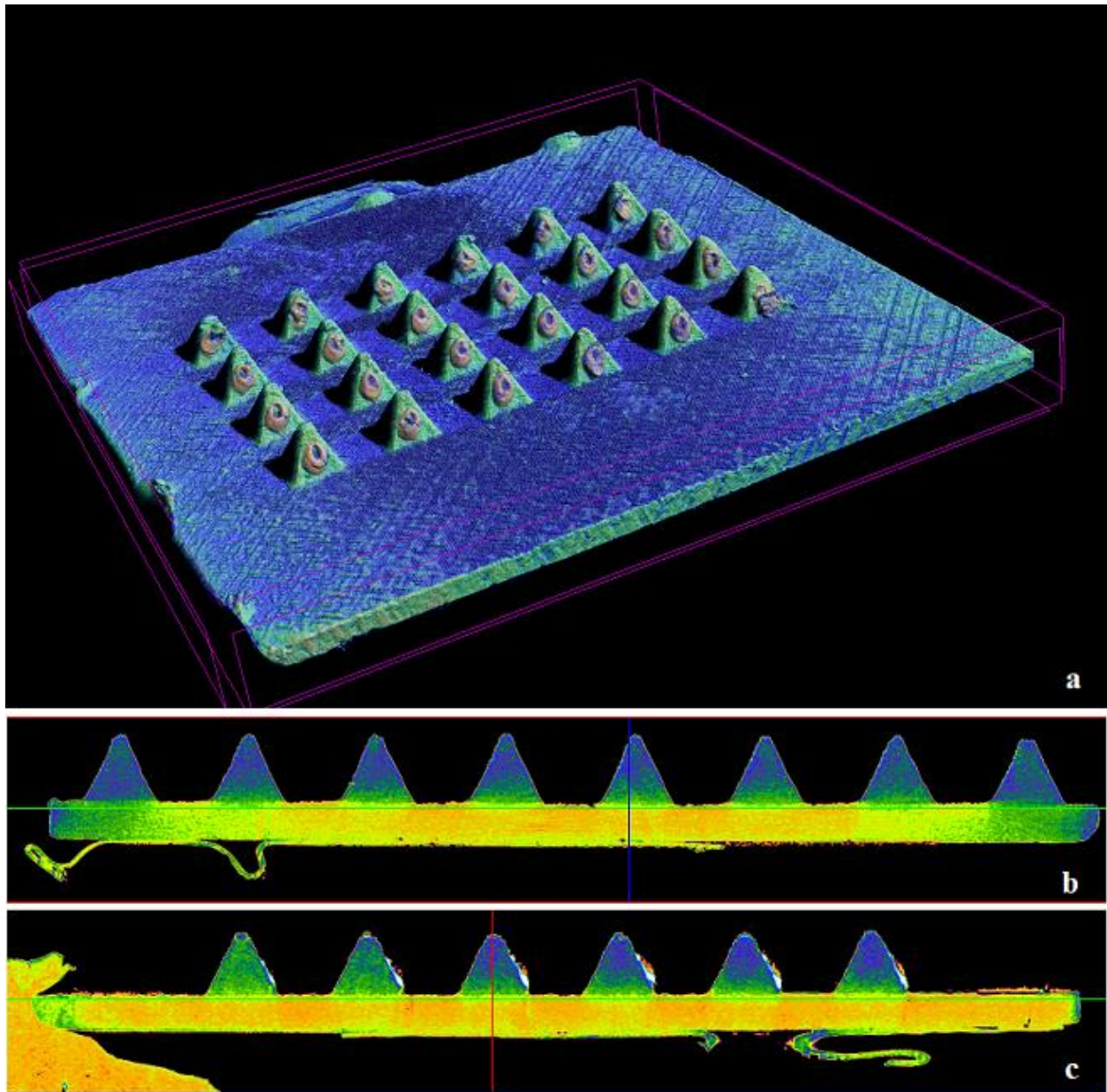
497 showing the optimum performance. *In vivo* animal trials demonstrated that 3D printed MNs
498 facilitate rapid low glucose levels with longer duration compared to SC injections.

500 FIGURES



501

502 Figure 1. SEM images of the 3D printed MNs. (a) Uncoated pyramid; (b) uncoated spear; (c)
503 coated pyramid; (d) coated spear. The thin coating films on the MNs were created using an inkjet
504 printer. The formulations employed for the coatings contained insulin and a sugar used as a
505 carrier (xylitol, mannitol and trehalose) in a 5:1 ratio. Each MN patch was coated with 10 IU. All
506 insulin-carrier combinations formed coatings with similar morphology. The carrier used for
507 captions (c) and (d) is xylitol.

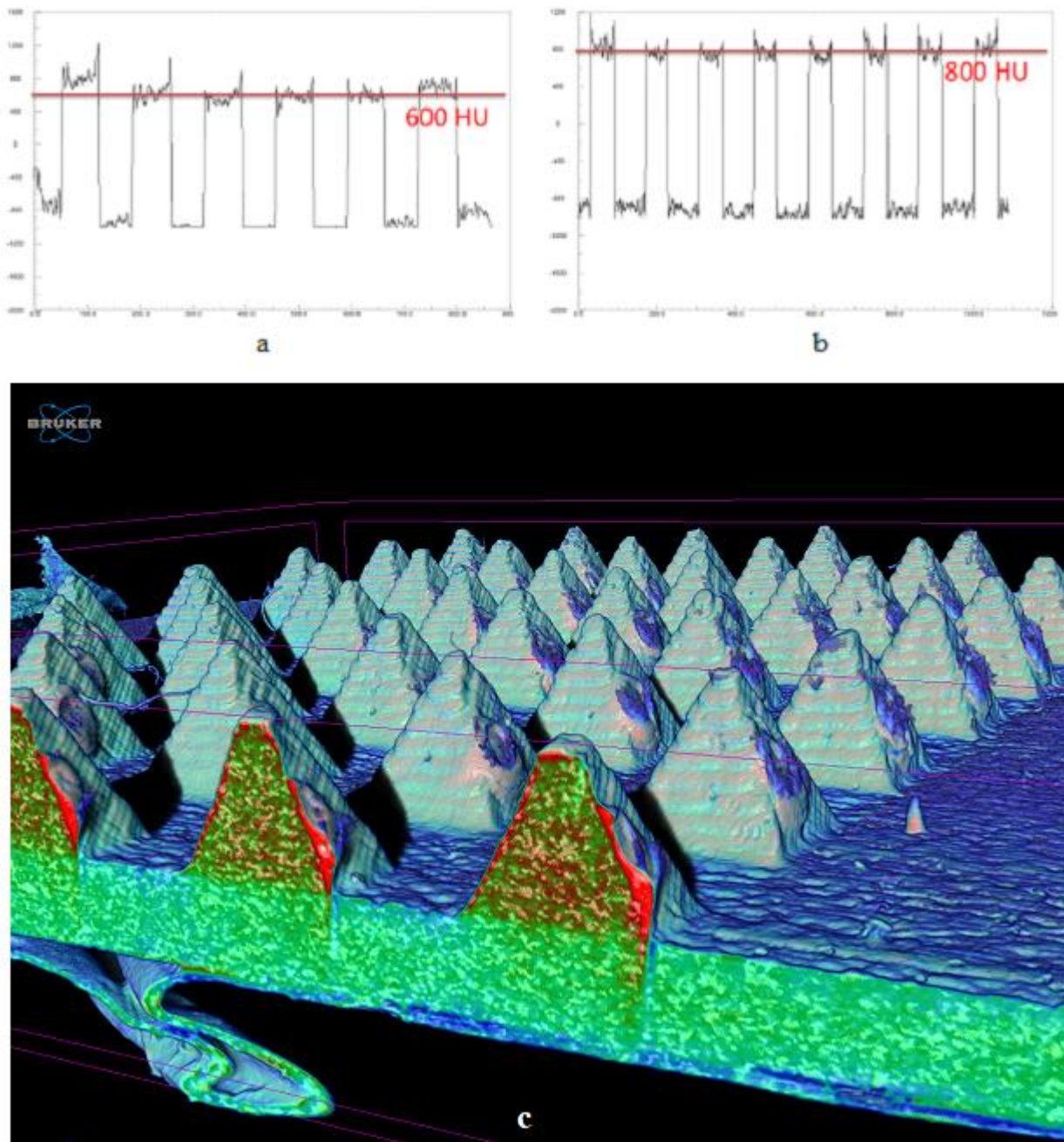


509

510 Figure 2. μ CT images of the pyramid MN arrays coated with insulin:xylitol formulation.

511 (a) Overview; (b) image taken from the back side; (c) image taken from the left-hand side of the

512 array, showing the thin film coating.

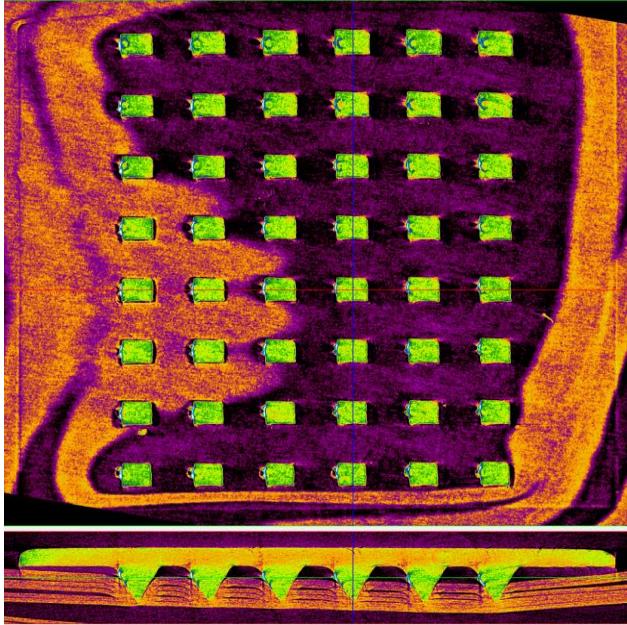


513

514 Figure 3. μ CT evaluation. (a) Profile lines, measured across a single row of the control sample

515 (uncoated array) and (b) the coated pyramid MN array with insulin:xylitol formulation, showing

516 a 200 HU increase; (c) cross-section of the coated MN array showing a fringe layer of 10-15 μ m.

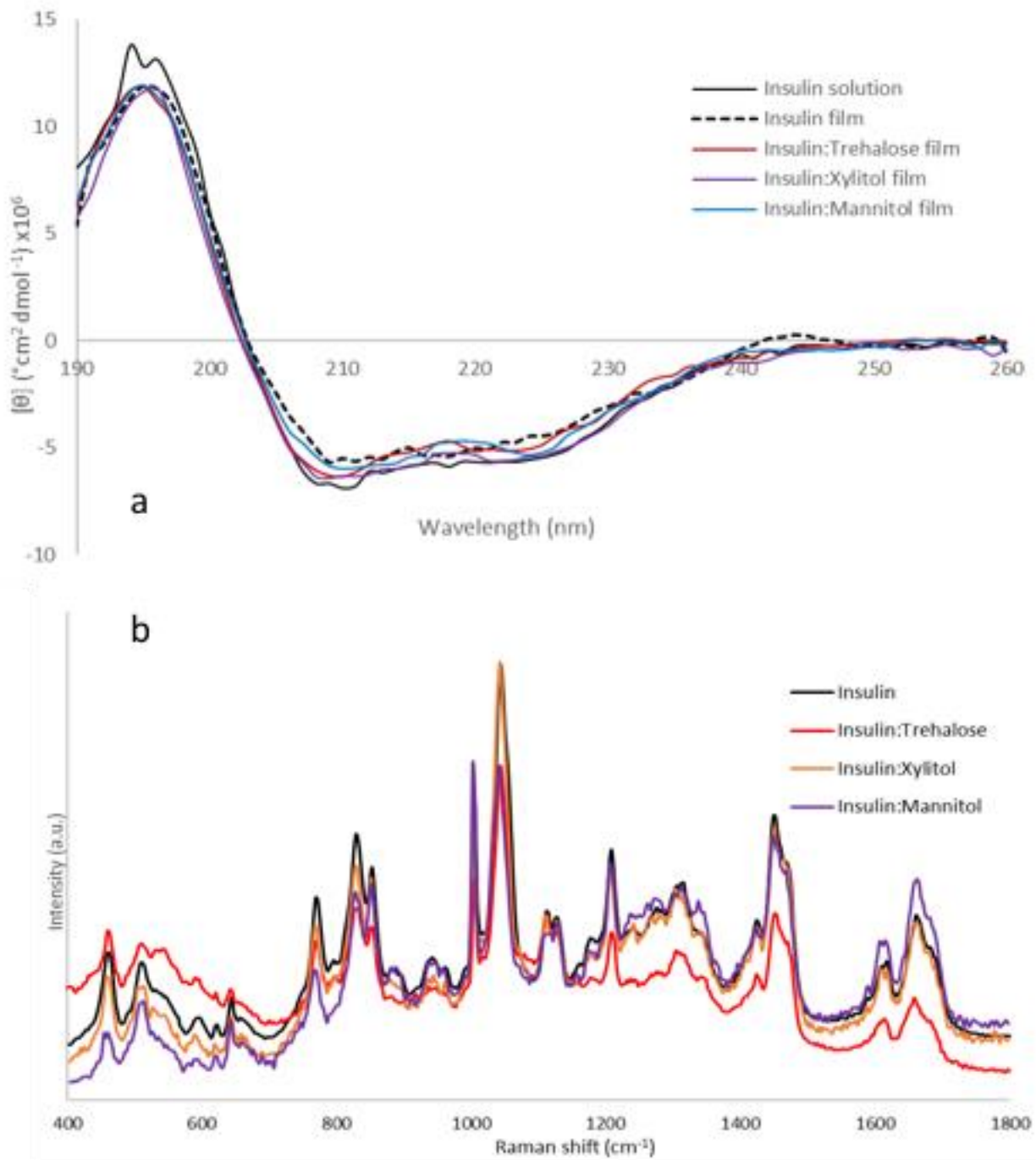


517

518 Figure 4. Cross section of MN array penetration through 8-ply strip of parafilm, applying a 5N
519 load.

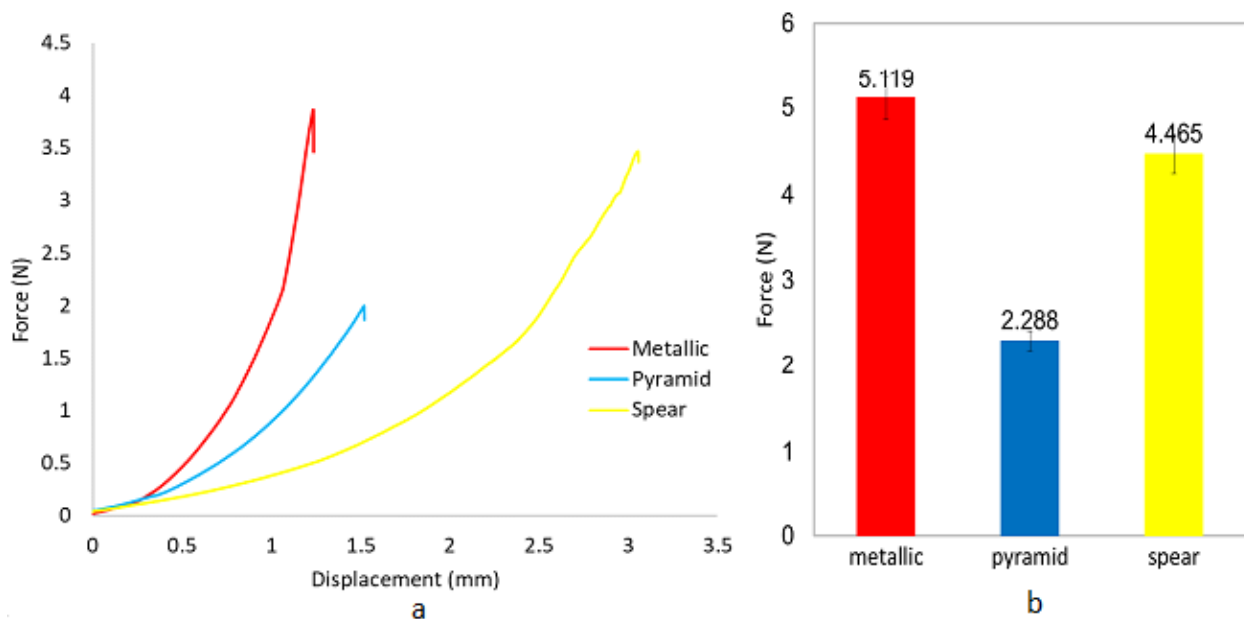
520

521
522

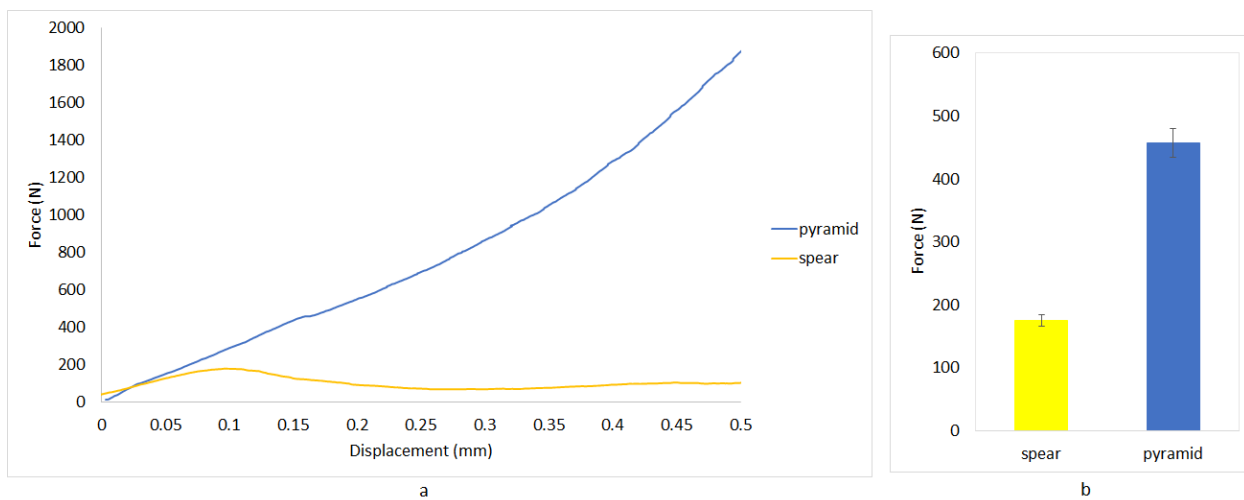


523

524 Figure 5. a) CD of insulin and insulin formulations and b) Raman spectra from 500 to 1800 cm^{-1}
525 of pure insulin and insulin-sugars.

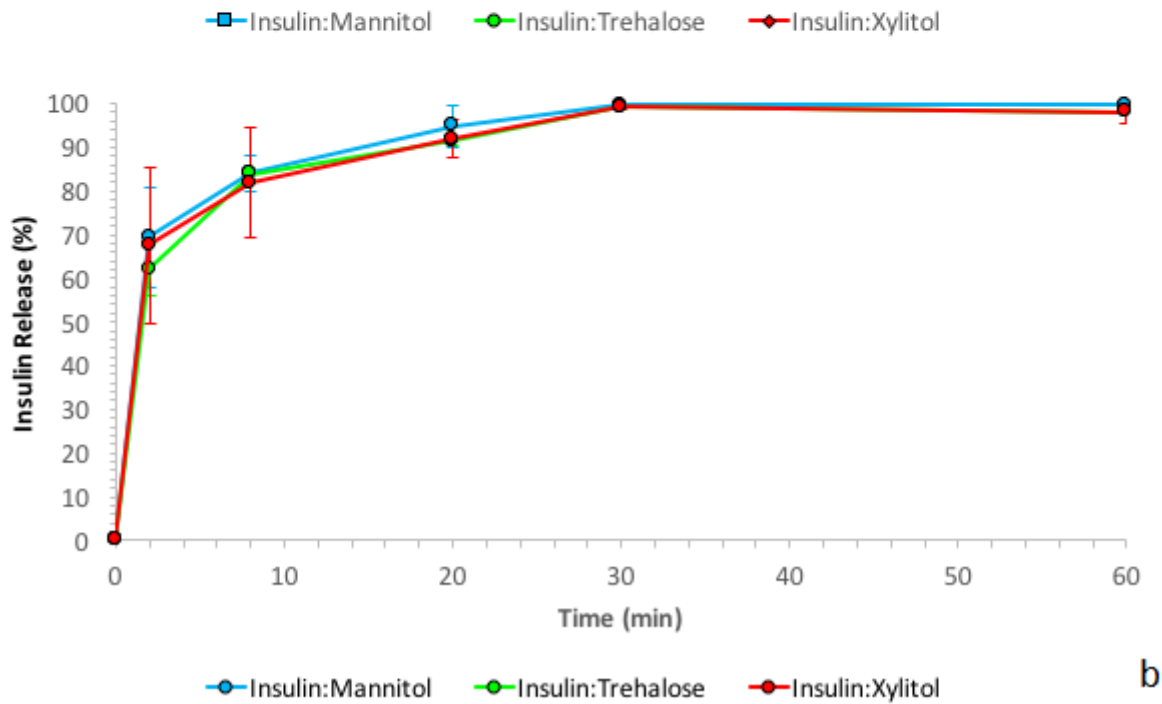
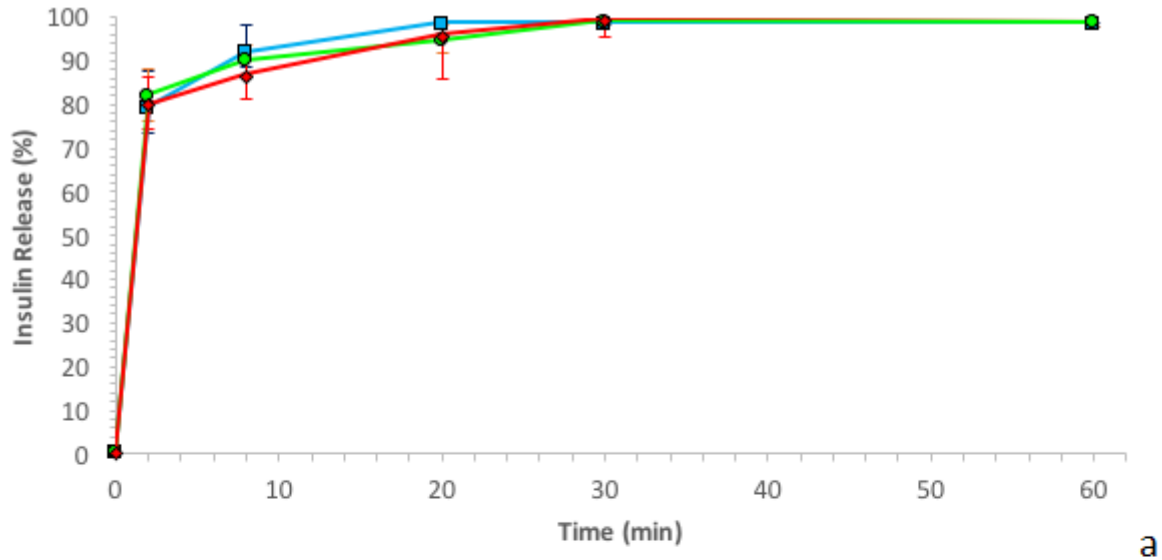


526
 527 Figure 6. Penetration studies of MNs through porcine skin, comparing 3D printed spear and
 528 pyramid designs with metallic MNs. (a) Force against displacement curves recorded during MN
 529 insertion tests; (b) Maximum force required for MN penetration.



530
 531 Figure 7. MN fracture testing for pyramid and spear designs. (a) Force against displacement
 532 curves recorded during MN fracture tests; (b) Fracture MN strength

533



534

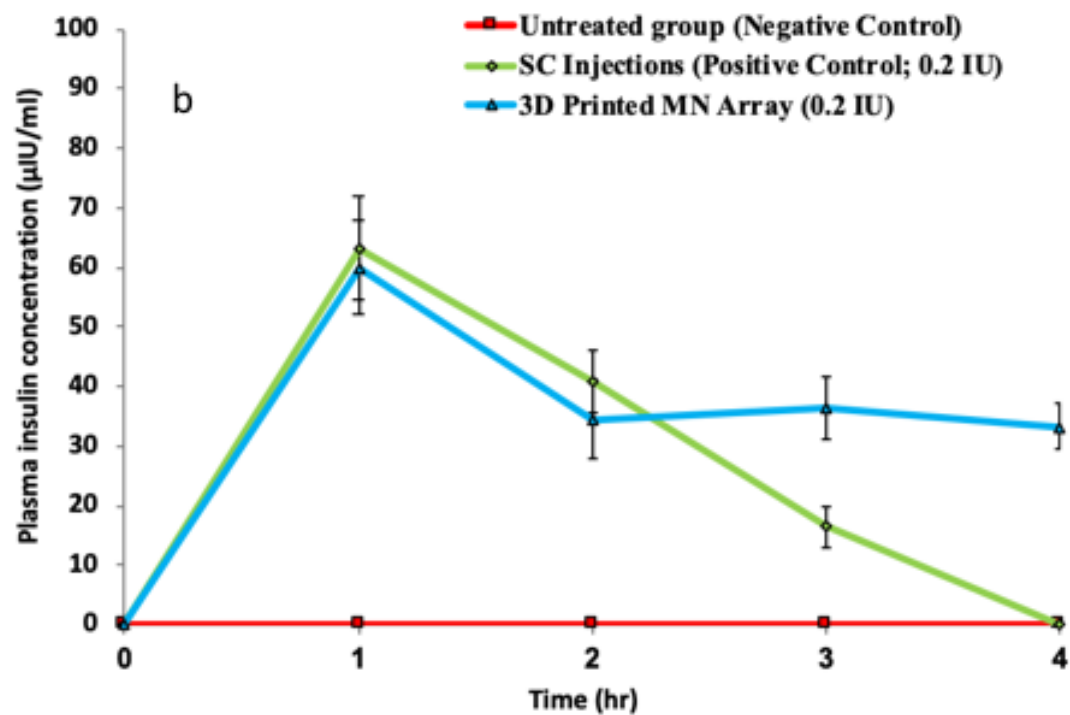
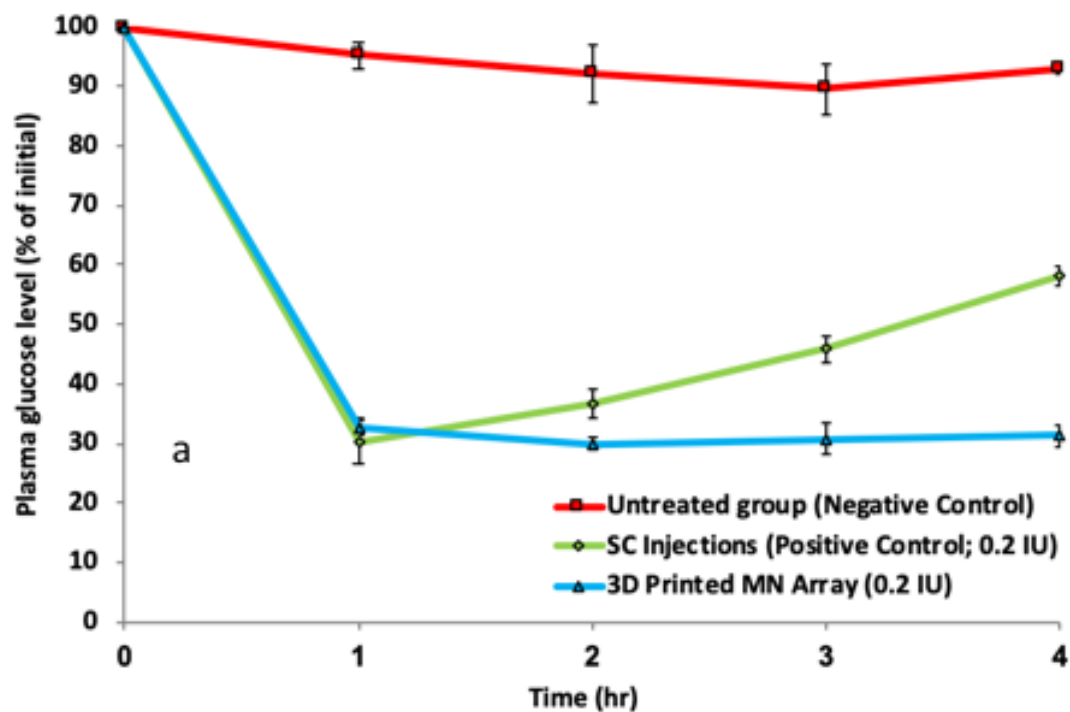
535 Figure 8. In vitro insulin release through porcine skin from a) the pyramid and b) the spear MN

536 designs for all investigated drug carriers.

537



538
539 Figure 9. Experimental mice (A) before the application, (B) during the application and (C) after
540 removal of the 3D printed MN array for the delivery of insulin to diabetic mice.



541
 542 Figure 10. a) Comparative plasma glucose level vs time for untreated group, subcutaneous (SC)
 543 injection and insulin-coated 3D Printed MN array applied to diabetic mice, over 4 hours (n=3),

544 b) comparative plasma insulin concentration vs time for untreated group, subcutaneous (SC)
 545 injection and insulin-coated 3D Printed MN array applied to diabetic mice, over 4 hours (n=3).

546

547 TABLES

548 **Table 1** Treatment protocol for insulin-coated 3D printed MN array.

Day	Stage	Treatment strategy
-7	Animal model selection and isolation	Transfer mice to study isolator
0	Induction of diabetes	<ul style="list-style-type: none"> • Weigh animals and injected with streptozotocin (diabetes inducer) • Daily blood glucose measurements
1		
2		
3	Observation	Blood glucose level measurements
4		
5		
6		
7	Confirmation of diabetes induction	Blood glucose level exceeds 300 mg/dl within 7 days.
8	Antidiabetic therapy using insulin-coated 3D printed MN array	<p>After treatment, hourly (up to 4 hours) blood samples were collected from the jugular vein and the blood glucose level was measured.</p> <p>The study was conducted for 24 hours.</p>

549

550

551 **Table 2.** Pharmacodynamic parameters for plasma glucose levels of diabetic mice for untreated
552 groups, subcutaneous (SC) injection (insulin dose: 0.2 IU) and 3D Printed MN array (insulin
553 dose: 0.2 IU) (n=3).

Group	C_{min} (%)	T_{min} (h)	AAC_{0 to 4} (% hr)	RPA (%)
Untreated groups	89.5 ± 4.2	3	22.7 ± 1.2	-
SC injections	32.8 ± 3.7	1	208.5 ± 3.7	100
3D Printed MN arrays	30.1 ± 1.0	1	240.6 ± 2.9	122

554 C_{min}, minimum glucose level; T_{min}, time point of minimum glucose level; AAC_{0 to 4}, area above
555 the plasma glucose concentration vs. time curve; RPA, relative pharmacological availability
556 compared to subcutaneous injection.

557

558 **Table 3.** Pharmacokinetic parameters for plasma glucose levels of diabetic mice for untreated
559 groups, subcutaneous (SC) injection (insulin dose: 0.2 IU) and 3D Printed MN array (insulin
560 dose: 0.2 IU) (n=3).

Group	C_{max} (μIU ml⁻¹)	T_{max} (h)	AUC_{0 to 4} (μIUhr⁻¹ ml⁻¹)	RBA (%)
Untreated groups	0	0	0	0
SC injections	63.2 ± 8.8	1	120.5 ± 6.4	100
3D Printed MN arrays	59.9 ± 7.9	1	147.4 ± 5.8	115

561 C_{max}, maximum plasma insulin concentration; T_{max}, time point of maximum plasma insulin
562 concentration; AUC_{0 to 4}, area under the plasma insulin concentration vs. time curve; RBA,
563 relative bioavailability compared with subcutaneous injection.

564

565 AUTHOR INFORMATION

566 **Corresponding Authors**

567 * Corresponding Authors: Prof. D.A. Lamprou, E-mail address: d.lamprou@qub.ac.uk, Tel.:
568 +44(0) 2890 97 2617. Prof. D. Douroumis, E-mail: d.douroumis@gre.ac.uk, Tel: +44 (0) 2083
569 31 8440.

570 **Author Contributions**

571 The manuscript was written through contributions of all authors. All authors have given approval
572 to the final version of the manuscript.

573 **Funding Sources**

574 This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
575 (CAPES) Foundation - Ministry of Education of Brazil; and the European Research Council
576 under the European Union's Seventh Framework Programme (FP/2007-2013) / ERC (grant
577 number. 615030).

578 ABBREVIATIONS

579 Transdermal Drug Delivery, TDD; MN, microneedle; poly(vinyl alcohol), PVA; glucose
580 oxidase, GOx; Additive Manufacturing, AM; UV, ultraviolet; CD, Circular Dichroism; μ CT,
581 Micro Computer Tomography; Scanning electron microscopy, (SEM); High-Performance Liquid
582 Chromatography, (HPLC); relative humidity, (RH).

583 DATA AVAILABILITY

584 The data will be available on request.

585

586

587 REFERENCES

588

- 589 [1] A.Z. Alkilani, M.T.C. McCrudden, R.F. Donnelly, Transdermal drug delivery: Innovative
590 pharmaceutical developments based on disruption of the barrier properties of the stratum
591 corneum, *Pharmaceutics*. 7 (2015) 438–470. doi:10.3390/pharmaceutics7040438.
- 592 [2] W. Chen, H. Li, D. Shi, Z. Liu, W. Yuan, Microneedles As a Delivery System for Gene
593 Therapy, *Front. Pharmacology*. 7 (2016) 137. doi:10.3389/fphar.2016.00137.
- 594 [3] S.T. Sanjay, W. Zhou, M. Dou, H. Tavakoli, L. Ma, F. Xu, X.J. Li, Recent advances of
595 controlled drug delivery using microfluidic platforms, *Adv. Drug Deliv. Rev.* 128 (2018)
596 3–28. doi:10.1016/j.addr.2017.09.013.
- 597 [4] L. Goodchild, Could dissolvable microneedles replace injected vaccines?, *Mater. Today*.
598 18 (2015) 419–420. doi:10.1016/j.mattod.2015.08.005.
- 599 [5] S. Kaushik, A.H. Hord, D.D. Denson, D. V. McAllister, S. Smitra, M.G. Allen, M.R.
600 Prausnitz, Lack of pain associated with microfabricated microneedles, *Anesth. Analg.* 92
601 (2001) 502–504. doi:10.1213/00000539-200102000-00041.
- 602 [6] D.P. Wermeling, S.L. Banks, D.A. Hudson, H.S. Gill, J. Gupta, M.R. Prausnitz, A.L.
603 Stinchcomb, Microneedles permit transdermal delivery of a skin-impermeant medication
604 to humans, *Proc. Natl. Acad. Sci.* 105 (2008) 2058–2063. doi:10.1073/pnas.0710355105.
- 605 [7] R.F. Donnelly, T.R.R. Singh, M.J. Garland, K. Migalska, R. Majithiya, C.M. McCrudden,
606 P.L. Kole, T.M.T. Mahmood, H.O. McCarthy, A.D. Woolfson, Hydrogel-forming
607 microneedle arrays for enhanced transdermal drug delivery, *Adv. Funct. Mater.* 22 (2012)
608 4879–4890. doi:10.1002/adfm.201200864.

- 609 [8] M. Korytkowski, L. Niskanen, T. Asakura, FlexPen®: Addressing issues of confidence
610 and convenience in insulin delivery, *Clin. Ther.* 27 (2005) S89-100.
611 doi:10.1016/j.clinthera.2005.11.019.
- 612 [9] X. Guo, W. Wang, Challenges and recent advances in the subcutaneous delivery of
613 insulin, *Expert Opin. Drug Deliv.* 14 (2017) 727–734.
614 doi:10.1080/17425247.2016.1232247.
- 615 [10] R.J. Narayan, Transdermal delivery of insulin via microneedles, *J. Biomed. Nanotechnol.*
616 10 (2014) 2244–2260. doi:10.1166/jbn.2014.1976.
- 617 [11] J. Wang, Y. Ye, J. Yu, A.R. Kahkoska, X. Zhang, C. Wang, W. Sun, R.D. Corder, Z.
618 Chen, S.A. Khan, J.B. Buse, Z. Gu, Core-Shell Microneedle Gel for Self-Regulated
619 Insulin Delivery, *ACS Nano.* 12 (2018) 2466–2473. doi:10.1021/acsnano.7b08152.
- 620 [12] W. Yu, G. Jiang, Y. Zhang, D. Liu, B. Xu, J. Zhou, Polymer microneedles fabricated from
621 alginate and hyaluronate for transdermal delivery of insulin, *Mater. Sci. Eng. C.* 80 (2017)
622 187–196. doi:10.1016/j.msec.2017.05.143.
- 623 [13] Y. Zhang, G. Jiang, W. Yu, D. Liu, B. Xu, Microneedles fabricated from alginate and
624 maltose for transdermal delivery of insulin on diabetic rats, *Mater. Sci. Eng. C.* 85 (2018)
625 18–26. doi:https://doi.org/10.1016/j.msec.2017.12.006.
- 626 [14] S. Ross, N. Scoutaris, D. Lamprou, D. Mallinson, D. Douroumis, Inkjet printing of insulin
627 microneedles for transdermal delivery, *Drug Deliv. Transl. Res.* 5 (2015) 451–461.
628 doi:10.1007/s13346-015-0251-1.
- 629 [15] R.D. Pedde, B. Mirani, A. Navaei, T. Styan, S. Wong, M. Mehrali, A. Thakur, N.K.
630 Mohtaram, A. Bayati, A. Dolatshahi-Pirouz, M. Nikkhah, S.M. Willerth, M. Akbari,
631 Emerging Biofabrication Strategies for Engineering Complex Tissue Constructs, *Adv.*

- 632 Mater. 29 (2017) 1–27. doi:10.1002/adma.201606061.
- 633 [16] L.E. Visscher, H.P. Dang, M.A. Knackstedt, D.W. Hutmacher, P.A. Tran, 3D printed
634 Polycaprolactone scaffolds with dual macro-microporosity for applications in local
635 delivery of antibiotics, Mater. Sci. Eng. C. 87 (2018) 78–89.
636 doi:10.1016/j.msec.2018.02.008.
- 637 [17] H. Tayebi, Lobat.; Rasoulianboroujeni, Morteza.; Moharamzadeh, Keyvan.; Almela,
638 Thafar.K.D.; Cui, Zhanfeng.; Ye, 3D-printed membrane for guided tissue regeneration,
639 Mater. Sci. Eng. C. 84 (2018) 148–158. doi:doi.org/10.1016/j.msec.2017.11.027.
- 640 [18] D. Lam, CXF; Mo, XM; Teoh, SH; Hutmacher, Scaffold development using 3D printing
641 with a starch-based polymer, Mater. Sci. Eng. C. 20 (2002) 49–56.
642 doi:doi.org/10.1016/S0928-4931(02)00012-7.
- 643 [19] S.N. Economidou, D.A. Lamprou, D. Douroumis, 3D printing applications for transdermal
644 drug delivery, Int. J. Pharm. 544 (2018) 415–424. doi:10.1016/j.ijpharm.2018.01.031.
- 645 [20] B. Thavornnyutikarn, P. Tesavibul, K. Sitthiseripratip, N. Chatarapanich, B. Feltis, P.F.A.
646 Wright, T.W. Turney, Porous 45S5 Bioglass®-based scaffolds using stereolithography:
647 Effect of partial pre-sintering on structural and mechanical properties of scaffolds, Mater.
648 Sci. Eng. C. 75 (2017) 1281–1288. doi:10.1016/j.msec.2017.03.001.
- 649 [21] D. Pede, G. Serra, D. De Rossi, Microfabrication of conducting polymer devices by ink-
650 jet stereolithography, Mater. Sci. Eng. C. 5 (1998) 289–291. doi:10.1016/S0928-
651 4931(97)00056-8.
- 652 [22] E.J. Mott, M. Busso, X. Luo, C. Dolder, M.O. Wang, J.P. Fisher, D. Dean, Digital
653 micromirror device (DMD)-based 3D printing of poly(propylene fumarate) scaffolds,
654 Mater. Sci. Eng. C. 61 (2016) 301–311. doi:10.1016/j.msec.2015.11.071.

- 655 [23] S.D. Gittard, P.R. Miller, C. Jin, T.N. Martin, R.D. Boehm, B.J. Chisholm, S.J. Stafslie,
656 J.W. Daniels, N. Cilz, N.A. Monteiro-Riviere, A. Nasir, R.J. Narayan, Deposition of
657 antimicrobial coatings on microstereolithography-fabricated microneedles, *Jom.* 63 (2011)
658 59–68. doi:10.1007/s11837-011-0093-3.
- 659 [24] Y. Lu, S.N. Mantha, D.C. Crowder, S. Chinchilla, K.N. Shah, Y.H. Yun, R.B. Wicker,
660 J.W. Choi, Microstereolithography and characterization of poly(propylene fumarate)-
661 based drug-loaded microneedle arrays, *Biofabrication.* 7 (2015) 1–13. doi:10.1088/1758-
662 5090/7/4/045001.
- 663 [25] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular
664 dichroism spectra: Comparison of CONTIN, SELCON, and CDSSTR methods with an
665 expanded reference set, *Anal. Biochem.* 287 (2000) 252–260.
666 doi:10.1006/abio.2000.4880.
- 667 [26] S.P. Davis, B.J. Landis, Z.H. Adams, M.G. Allen, M.R. Prausnitz, Insertion of
668 microneedles into skin: Measurement and prediction of insertion force and needle fracture
669 force, *J. Biomech.* 37 (2004) 1155–1163. doi:10.1016/j.jbiomech.2003.12.010.
- 670 [27] M.J. Uddin, N. Scoutaris, P. Klepetsanis, B. Chowdhry, M.R. Prausnitz, D. Douroumis,
671 Inkjet printing of transdermal microneedles for the delivery of anticancer agents, *Int. J.*
672 *Pharm.* 494 (2015) 593–602. doi:10.1016/j.ijpharm.2015.01.038.
- 673 [28] Z. Yong, D. Yingjie, W. Xueli, X. Jinghua, L. Zhengqiang, Conformational and
674 bioactivity analysis of insulin: Freeze-drying TBA/water co-solvent system in the
675 presence of surfactant and sugar, *Int. J. Pharm.* 371 (2009) 71–81.
676 doi:10.1016/j.ijpharm.2008.12.018.
- 677 [29] Y.H. Kim, C. Sioutas, K.S. Shing, Influence of stabilizers on the physicochemical

678 characteristics of inhaled insulin powders produced by supercritical antisolvent process,
679 Pharm. Res. 26 (2009) 61–71. doi:10.1007/s11095-008-9708-y.

680 [30] H. Schiffter, J. Condliffe, S. Vonhoff, Spray-freeze-drying of nanosuspensions: the
681 manufacture of insulin particles for needle-free ballistic powder delivery, J. R. Soc.
682 Interface. 7 (2010) S483-500. doi:10.1098/rsif.2010.0114.focus.

683 [31] L. Whitmore, B.A. Wallace, DICHROWEB, an online server for protein secondary
684 structure analyses from circular dichroism spectroscopic data, Nucleic Acids Res. 32
685 (2004) 668–673. doi:10.1093/nar/gkh371.

686 [32] L. Whitmore, B.A. Wallace, Protein secondary structure analyses from circular dichroism
687 spectroscopy: Methods and reference databases, Biopolymers. 89 (2008) 392–400.
688 doi:10.1002/bip.20853.

689 [33] M.J. Ettinger, S.N. Timasheff, Optical activity of insulin. II. Effect of nonaqueous
690 solvents, Biochemistry. 10 (1971) 831–840.
691 http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=5544674.
692

693 [34] B. Sarmiento, D.C. Ferreira, L. Jorgensen, M. van de Weert, Probing insulin's secondary
694 structure after entrapment into alginate/chitosan nanoparticles, Eur. J. Pharm. Biopharm.
695 65 (2007) 10–17. doi:10.1016/j.ejpb.2006.09.005.

696 [35] F. Andrade, P. Fonte, M. Oliva, M. Videira, D. Ferreira, B. Sarmiento, Solid state
697 formulations composed by amphiphilic polymers for delivery of proteins: characterization
698 and stability, Int. J. Pharm. 486 (2015) 195–206. doi:10.1016/j.ijpharm.2015.03.050.

699 [36] W. Dzwolak, R. Ravindra, J. Lendermann, R. Winter, Aggregation of bovine insulin
700 probed by DSC/PPC calorimetry and FTIR spectroscopy, Biochemistry. 42 (2003) 11347–

701 11355. doi:10.1021/bi034879h.

702 [37] J.F. Carpenter, J.H. Crowe, An infrared spectroscopic study of the interactions of
703 carbohydrates with dried proteins, *Biochemistry*. 28 (1989) 3916–3922.
704 doi:10.1021/bi00435a044.

705 [38] M.A. Haque, J. Chen, P. Aldred, B. Adhikari, Drying and denaturation characteristics of
706 whey protein isolate in the presence of lactose and trehalose, *Food Chem.* 177 (2015) 8–
707 16. doi:10.1016/j.foodchem.2014.12.064.

708 [39] S.G. Melberg, W.C. Johnson, Changes in secondary structure follow the dissociation of
709 human insulin hexamers: A circular dichroism study, *Proteins Struct. Funct. Bioinforma.* 8
710 (1990) 280–286. doi:10.1002/prot.340080309.

711 [40] D.C.O. Nai-Teng Yu, C.S. Liu, Laser Raman Spectroscopy and the Conformation and
712 Proinsulin of Insulin, *J. Mol. Biol.* 70 (1972) 117–132.

713 [41] P.O. Souillac, C.R. Middaugh, J.H. Rytting, Investigation of protein / carbohydrate
714 interactions in the dried state . 2 . Diffuse reflectance FTIR studies, *Int. J. Pharm.* 235
715 (2002) 207–218.

716 [42] A. Das, P. Basak, R. Pattanayak, T. Kar, R. Majumder, D. Pal, A. Bhattacharya, M.
717 Bhattacharyya, S.P. Banik, Trehalose induced structural modulation of Bovine Serum
718 Albumin at ambient temperature, *Int. J. Biol. Macromol.* 105 (2017) 645–655.
719 doi:10.1016/j.ijbiomac.2017.07.074.

720 [43] J. Lee, S. Timasheff, The Stabilization of Proteins by Sucrose *, *J. Biol. Chem.* 256
721 (1981) 7193–7201.

722 [44] S. Yoshioka, T. Miyazaki, Y. Aso, b-Relaxation of Insulin Molecule in Lyophilized
723 Formulations Containing Trehalose or Dextran As a Determinant of Chemical Reactivity,

724 Pharm. Res. 23 (2006) 961–966. doi:10.1007/s11095-006-9907-3.

725 [45] C. Branca, S. MacCarrone, S. Magazu, G. Maisano, S.M. Bennington, J. Taylor,
726 Tetrahedral order in homologous disaccharide-water mixtures, *J. Chem. Phys.* 122 (2005)
727 174513-1-174513–6. doi:10.1063/1.1887167.

728 [46] N.K. Jain, I. Roy, Effect of trehalose on protein structure, *Protein Sci.* 18 (2009) 24–36.
729 doi:10.1002/pro.3.

730 [47] G. Zeng, J.J. Shou, K.K. Li, Y.H. Zhang, In-situ confocal Raman observation of structural
731 changes of insulin crystals in sequential dehydration process, *Biochim. Biophys. Acta -*
732 *Proteins Proteomics.* 1814 (2011) 1631–1640. doi:10.1016/j.bbapap.2011.09.002.

733 [48] L.G. Tensmeyer, J.E. Shields, E. Lilly, The Raman Spectra of Crystalline 4Zn, 2Zn, and
734 Na Insulin., 1336 (1990) 222–234.

735 [49] H. Takeuchi, N. Watanabe, Y. Satoh, I. Harada, Effects of Hydrogen Bonding on the
736 Tyrosine Raman Bands in the 1300-1150 cm⁻¹ Region, 20 (1989) 233–237.

737 [50] S.D. Gittard, B. Chen, H. Xu, A. Ovsianikov, B.N. Chichkov, N.A. Monteiro-Riviere, R.J.
738 Narayan, The effects of geometry on skin penetration and failure of polymer
739 microneedles, *J. Adhes. Sci. Technol.* 27 (2013) 227–243.
740 doi:10.1080/01694243.2012.705101.

741 [51] I.C. Lee, Y.C. Wu, S.W. Tsai, C.H. Chen, M.H. Wu, Fabrication of two-layer dissolving
742 polyvinylpyrrolidone microneedles with different molecular weights for: In vivo insulin
743 transdermal delivery, *RSC Adv.* 7 (2017) 5067–5075. doi:10.1039/c6ra27476e.

744 [52] J. Gupta, S.S. Park, B. Bondy, E.I. Felner, M.R. Prausnitz, Infusion pressure and pain
745 during microneedle injection into skin of human subjects, *Biomaterials.* 32 (2011) 6823–
746 6831. doi:10.1016/j.biomaterials.2011.05.061.

- 747 [53] M. Ling, M. Chen, Dissolving polymer microneedle patches for rapid and efficient
748 transdermal delivery of insulin to diabetic rats., *Acta Biomater.* 9 (2013) 8952–8961.
749 doi:10.1016/j.actbio.2013.06.029.
- 750 [54] S. Liu, M. Jin, Y. Quan, F. Kamiyama, H. Katsumi, T. Sakane, A. Yamamoto, The
751 development and characteristics of novel microneedle arrays fabricated from hyaluronic
752 acid, and their application in the transdermal delivery of insulin., *J. Control. Release.* 161
753 (2012) 933–41. doi:10.1016/j.jconrel.2012.05.030.
- 754 [55] S. Fakhraei Lahiji, Y. Jang, I. Huh, H. Yang, M. Jang, H. Jung, Exendin-4-encapsulated
755 dissolving microneedle arrays for efficient treatment of type 2 diabetes, *Sci. Rep.* 8 (2018)
756 1–9. doi:10.1038/s41598-018-19789-x.
- 757 [56] Y. Qiu, G. Qin, S. Zhang, Y. Wu, B. Xu, Y. Gao, Novel lyophilized hydrogel patches for
758 convenient and effective administration of microneedle-mediated insulin delivery, *Int. J.*
759 *Pharm.* 437 (2012) 51–56. doi:10.1016/j.ijpharm.2012.07.035.
- 760 [57] S.P. Davis, W. Martanto, M.G. Allen, S. Member, M.R. Prausnitz, Hollow Metal
761 Microneedles for Insulin Delivery to Diabetic Rats, 52 (2005) 909–915.
762