Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors are commended for devising a series of processing methods to approach the multilayered design of the artery with control of the stress-strain curve. In this respect, the accomplishment seems substantial. However, for impact in the field, sufficient strength is required, and these constructs lack it: there is no explicit mention of "strength" that I could find in terms of results presented or how the approach could be modified to achieve it (not that the latter in and of itself would warrant publication in NCOMMS). But inspection of the stress-strain curves, assuming they are plotted to failure, shows the UTS is ~200KPa, about a factor of 10 below the physiological value. Even if 2MPa were achieved, physiological burst pressure would have to be demonstrated to show potential. And for publication in NCOMMS, I would expect beyond that a successful implantation study in the arterial circulation, not subcutaneous as presented here.

Reviewer #2 (Remarks to the Author):

This is an intriguing paper that reports a process for the construction of replacement arteries that effectively mimic the mechanical characteristics of native arteries. The authors describe their innovative fabrication process and show that cells included in the resulting vessels continue to be functional. While the reported methods and results are exciting, there are numerous problems with grammar, word choice, and punctuation that sometimes make it difficult to understand the authors' intent; aside from this, the paper is outstanding. Specific issues to be addressed are described below.

Many of the figures (see 3, 4, and 5) and their associated captions are confusing since the authors don't specify the meaning of all of the curves included in the plots and/or the caption descriptions don't seem to accurately correspond with the content of the plots. These need to be checked and corrected for better comprehension. Also

On line 174, the authors describe a pre-stretching step required to produce the characteristic J-shape of arteries, though little detail is given. It is not clear whether this was done during the fabrication itself, on a layer-by-layer basis as the vessel was formed, or just at the end of the fabrication process. This needs to be clarified. The paper should also specify and justify the extent of the pre-stretch utilized.

It is not clear why the authors chose to perform uniaxial strip tests on the fabricated vessel material, instead of simply testing biaxial behavior. This should be justified.

Table 1 does not appear to be referenced in the text of the manuscript

It is not clear that the cells used in the fabrication are relevant. Why weren't vascular cells (i.e. endothelial cells, smooth muscle cells, fibroblasts) used?

Please describe the details of the vessel culture from days 1 to 7.

Cell survival appears to be equated to function, but it should be made clear that vessel function (e.g. contractility) was not tested in this paper.

SIVG does not appear to be defined anywhere in the paper

Reviewer #3 (Remarks to the Author):

Title: Rapid fabrication of reinforced and cell-laden vascular grafts structurally inspired by human coronary arteries.
Authors: Akentjew, T.L., et al.

General Comments:
This is an interesting paper describing a combination of spinning and hydrogel technologies, that can deposit concentric layers of cell-laden hydrogels that are interspersed with re-inforcing fibers to provide mechanical strength.
Cell layers are contained in a methacryloyl gelatin-alginate (GEAL) substrate, while the reinforcing fibers are made from slow-degrading polycaprolactone (PCL). The idea of interspersing mechanically reinforcing fibers within a living tissue is novel and potentially important. However, this paper has multiple drawbacks that limit enthusiasm substantially. Overall, important information is lacking on the process, and the figures contain many errors and substantially lack clarity. Missing important information includes: what is the diameter of the grafts? How long does the graft manufacturing process take – can cells survive this period? Is the process sterile? How big are the PCL fibers? This is difficult to infer from the paper. What is the phenotype or appearance of the cells in the construct? There is zero histological information. How were MSC harvested and characterized? No information on this either.
Also, there is overall a lack of care in preparation of the paper. The figure legends contain many errors, the statistical analysis is uneven and poor overall, and the Methods descriptions do not comport with the data in the paper. Overall, English grammar should be improved as well.

Specific Comments:
1. Line 123: the statement is made that the GEAL solution is pre-crosslinked, but then the authors state that the dipped layer is exposed to UV light to crosslink the material. please clarify.
2. The phrase "circumferential axis" is used throughout the paper and is unclear to this reviewer.
3. After spraying the PCL layers, do the fibers fuse at the points of contact? What is the data for or against fiber fusion, which will significantly impact the vessel mechanical properties? What is the fiber diameter, is it uniform, how is it controlled?
4. The legend for Figure 4 is very confusing – panels a-c are stated as having data in the grey lines, but these lines are identical across the images, and the dotted lines are not described at all. It is pretty impossible to tell what this figure is saying. Do the authors have data to show on actual stress-strain curves of native human coronary arteries?
5. Similarly the legend for Figure 5 makes no sense – the grey lines and solid squares that are talked about the in the legend simply aren't there in the figure. And, panels b-e contain multiple black lines of unclear significance- if these are replicates, then should not some error bars be included or something? In Figures 5 and 7, where is the "grey region of the range of the model of the mechanical properties" derived from? This is not at all clear to the reader, and serves only to confuse.
6. In Table 1, what is the "n" value for these data? This bears on the statistical significance and differences.
7. Figure 8 needs scale bars. What is the meaning of the blue color – does this signal live or dead cells or proliferating cells? What are the dimensions of the construct? The reagent used does not show cell replication per se – it appears to show mitochondrial activity??
8. Figure 9 shows cells counts from histology, but there are no histological images shown in the main paper. This is very disappointing.
9. In the Methods section, the Histology paragraph talks about skin transplants and positive controls for immunity, and talks about FACS analysis using different markers for immune cells, but none of this data is shown in the paper. It appears as if this text was taken from another manuscript, since it does not match up with the data in this paper?

1   Reviewers' comments:

2

3   Reviewer #1 (Remarks to the Author):

4

5   The authors are commended for devising a series of processing methods to approach the

6   multilayered design of the artery with control of the stress-strain curve. In this respect, the

7   accomplishment seems substantial. However, for impact in the field, sufficient strength is

8   required, and these constructs lack it: there is no explicit mention of "strength" that I could

9   find in terms of results presented or how the approach could be modified to achieve it (not

10   that the latter in and of itself would warrant publication in NCOMMS). But inspection of the

11   stress-strain curves, assuming they are plotted to failure, shows the UTS is ~200KPa, about a

12   factor of 10 below the physiological value. Even if 2MPa were achieved, physiological burst

13   pressure would have to be demonstrated to show potential. And for publication in NCOMMS,

14   I would expect beyond that a successful implantation study in the arterial circulation, not

15   subcutaneous as presented here.

16

17

18   Response: We agree with the reviewer and have conducted experiments to assess questions

19   about the strength characterization and evaluation of physiological burst pressure of the

20   constructs. In these regards, sufficient ultimate tensile strength (UTS) was evaluated by

21   longitudinal tensile testing until fracture of the small diameter vascular graft (SDVG), whereas

22 for physiological burst pressure assessment pressurization testing of SDVGs were conducted

23 until failure.

24 In our first draft, we initially performed tensile tests using a fixed upper limit of strain to

25 evaluate mainly the stress-strain J-shape curve, without recording the UTS. Therefore, as well

26 mentioned by the reviewer, a reader could assume that results were plotted to failure. This is

27 now clarified in the text and material and methods. In the main text, Line 313 the following is

28 stated: "The construct exhibited mechanical response similar to human coronary arteries,

29 both in longitudinal and circumferential directions under uniaxial tensile testing up to a 30%

30 strain range (Fig. 7a-b).", and additionally, in material an methods line 781, the following is

31 mentioned: "Uniaxial tensile testing was performed at a constant strain rate of 1 mm/min and

32 up to 30% strain (see Fig. S2b)." However, since the failure data is relevant for the application

33 of this technology, as stated by the reviewer, we included information about the tensile

34 testing

35 to failure and physiological burst pressure results in the main text and supporting material.

36 We have summarized these results in line 315 for the resistance until failure: "Longitudinally

37 tested SDVG exhibited a maximum failure strength of 520 ± 56 kPa, which is in the range of

38 coronary arteries of individual above 35 years old[18,45] (Fig S4)". In line 363 the following results

39 concerning burst pressure were included: "Due to safety and clinical concerns, vascular grafts

40 must also meet adequate burst pressures and suture retention strength[25,47] .The fabricated

41 full SDVGs exhibited burst pressures of 1630 ± 180 mmHg, similar to values reported for

42 human saphenous vein, as well used as autologous graft for coronary bypass[48] (see Fig S5).".

43 Being the suture retention strength a particularly important aspect of vascular grafts in

44 regards to safety and clinical practice, we have included in line 366 additional results of suture

45   retention strength: "Support retention strength was determined at 143 ± 13 grams-force for

46   SDVGs, similar to reported values for human internal mammary artery (IMA)47(see Fig S6)".

47   We agree with the reviewer, concerning the need of successful implantation study in the

48   arterial circulation. To address this issue, we have included a whole new chapter in line 501

49   title "Implantability study in arterial circulation using a rabbit model". A preliminary study for

50   artery grafting was conducted in a rabbit carotid model (1-month period and patency test)

51   using a total of 6 rabbits, two with artery to artery anastomosis as surgery control, two with

52   acellularized SDVG and two with BM-MSCs-laden SDVG. During surgery, the grafting

53   experiments showed good suturing performance, no blood leakage observed, absence of

54   apparent immunological rejection or inflammation of the implanted grafts and absence of any

55   unexpected and adverse events during grafting. Except for the anastomosis control all

56   implanted rabbits showed SDVG patency between 12 h and 36 h. Grafting results are

57   discussed in line 623-640. We clarified in the discussion too that our SDVG design and iterative

58   improvement was originally directed to human coronary arteries. Nevertheless, the rabbit

59   model is an appropriate model to evaluate adverse effect derived from thrombosis and

60   immunorejection. In order to evaluate the SDVG in a rabbit carotid model, we modified the

61   SDVG design in terms of diameter, but not wall thickness and mechanical properties to match

62   that of the rabbit carotids. Therefore, the rabbit carotid model has certain limitation to assess

63   the grafting and bypass potential of the new SDVG, which are discuss in the manuscript (Lines

64   625-640). Additionally, in the revised version of the manuscript, we discuss the necessity for

65   testing on larger animal models, though we deem such tests out of the scope of the current

66   work, and indeed such tests will require significant further funding and time.

67 Additional to the implantability study, we have added *in vitro* hemocompatibility results (lines

68 451-499) and suturing retention strength (previously addressed in this response) to further

69 support arterial grafting potential.

70 Taking into consideration the issue raised with respect of the subcutaneous implantation by

71 the reviewer, we have further clarified the objective of that experimentation in the actual

72 manuscript. For example, in lines 586-593 ("Cells remained viable during fabrication, and

73 demonstrated proliferative and functional capacity within the SDVG (Fig. 8 and 9). In vivo

74 immunogenicity experiments demonstrate that the encapsulation of BM-MSCs lowers the

75 rejection and inflammation response cause by the subcutaneous implantation of an

76 endotoxin-laden SDVG. The immunosupression cell function is of importance in the design of a

77 new vascular graft, considering a cause of graft failure is associated to chronic inflammatory

78 response[5]. These results prove the importance of fabricating MSC-cellularized grafts to

79 prevent rejection and enhance the functionality of the implant by promoting cell repopulation

80 and definitive tissue remodeling after implantation[74].") we discuss that the subcutaneous

81 implantation model was used to evaluated the functional competence of encapsulated BM-

82 MSCs, suggesting that cells are still functionally viable after the manufacturing process, but as

83 well to demonstrated that BM-MSCs in the SDVG design could ameliorate inflammatory

84 responses derived from graft implantation, which has been associated as one cause of

85 vascular graft failure in clinic.

86

87 Reviewer #2 (Remarks to the Author):

88

89 This is an intriguing paper that reports a process for the construction of replacement arteries

90   that effectively mimic the mechanical characteristics of native arteries. The authors describe

91   their innovative fabrication process and show that cells included in the resulting vessels

92   continue to be functional. While the reported methods and results are exciting, there are

93   numerous problems with grammar, word choice, and punctuation that sometimes make it

94   difficult to understand the authors' intent; aside from this, the paper is outstanding. Specific

95   issues to be addressed are described below.

96

97   Many of the figures (see 3, 4, and 5) and their associated captions are confusing since the

98   authors don't specify the meaning of all of the curves included in the plots and/or the caption

99   descriptions don't seem to accurately correspond with the content of the plots. These need to

100 be checked and corrected for better comprehension.

101 Response: We thank the reviewer for his helpful comments. All figures and figure captions

102 have been revised accordingly. We have revised the entire manuscript for improved clarity

103 and to address the English throughout.

104 Also on line 174, the authors describe a pre-stretching step required to produce the

105 characteristic J-shape of arteries, though little detail is given. It is not clear whether this was

106 done during the fabrication itself, on a layer-by-layer basis as the vessel was formed, or just at

107 the end of the fabrication process. This needs to be clarified. The paper should also specify

108 and justify the extent of the pre-stretch utilized.

109 Response: We thank the reviewer for pointing this out. We have clarified the rationale behind

110 and use of the pre-stretching step, hereon called preconditioning step. We now include in line

111 206 the following: "In an effort to match the J-shape stress-strain curves[18], a series of 24 PCL

112   fibre sublayers were deposited at +/-21° and preconditioned after fabrication by stretching

113   cycles before subjecting the construct to longitudinal tensile testing.", and in the materials

114   and methods section in line 767 the following was included: "The preconditioning

115   loading/unloading cycles of the outer graft layer in the longitudinal and circumferential

116   direction were conducted to a strain level of 13% and 30%, respectively. Differences in strain

117   level were iteratively adjusted to obtain the target J-shape strain-stress curves. In the case of

118   the middle graft layer, the strain for preconditioning was 35% and 30% in the circumferential

119   and longitudinal tensile testing, respectively. Uniaxial testing for both, circumferential and

120   longitudinal samples, were performed at a constant rate of 10 mm/min (see Fig. S2a).".

121   It is important to understand the effect of angled fibre deposition on mechanical properties in

122   each of the layers and their pre-conditioning' tests were devised to assess this at the level of

123   the outer graft layer, middle graft layer and complete SDVG construct, as clarified in the text

124   (Lines 234-236: "In order to define the structural configuration of the middle and outer graft

125   layers of the new bio-inspired small diameter vascular graft (SDVG), iterative testing of

126   differently layered constructs was performed in an effort to match the stress-strain profiles of

127   the media and adventitia layers of human coronary arteries[15]" and lines 311-315: "Nonlinear

128   and anisotropic mechanical response are maintained when tested together in the

129   configuration of a full SDVG, comprising of inner, middle and outer graft layers, GEAL

130   sublayers, with wavy, orientated PCL fibre sublayers and preconditioning. The construct

131   exhibited mechanical response similar to human coronary arteries, both in longitudinal and

132   circumferential directions under uniaxial tensile testing up to a 30% strain range (Fig. 7a-b).")

133   Briefly, for the outer, middle graft layer and complete SDVG formulations, prior to mechanical

134   testing and after fabrication, layers were submitted to the pre-conditioning step (5 cycles of

6

135 30% strain). Beyond this, we have conducted pressurization testing, in which SDVG were

136 previously submitted to 5 cycles of 30% strain in the longitudinal direction after being

137 mounted cylindrically in the pressurization system coupled to the tensile machine (see

138 material and methods line 796) and 5 cycles of 200 mmHG pressure loading prior to test. In an

139 *in vivo* or clinical implantation setting, the pre-conditioning step may be obviated, although

140 this has not been addressed in the actual study (line 548: "It may even be possible to conduct

141 the preconditioning in situ on implantation by pulsatile blood pressure without need of

142 previous preconditioning and is the subject of future studies.").

143

144 It is not clear why the authors chose to perform uniaxial strip tests on the fabricated vessel

145 material, instead of simply testing biaxial behavior. This should be justified.

146 Response: Indeed, biaxial mechanical testing would be more appropriate for mechanical

147 analysis of vasculature tissues, as mentioned by the reviewer, especially as this type of tissue

148 is submitted to biaxial stretching during pulsatile blood flow. However, and since our study

149 strongly rely on previous experimental and theoretical studies based on uniaxial testing

150 analysis, we decided to use equivalent experimental setting to guide our fabrication design in

151 order to match the native mechanics.

152

153 Table 1 does not appear to be referenced in the text of the manuscript

154 Response: We thanks the reviewer for pointing this out. Table 1 is now referenced in the new

155 manuscript in line 354 Quoted here: "Compliance values for SDVGs at 10% and 20% of axial

156 stretching condition showed no statistical difference with reported results for human

157   coronary arteries (Table 1)."

158

159   It is not clear that the cells used in the fabrication are relevant. Why weren't vascular cells (i.e.

160   endothelial cells, smooth muscle cells, fibroblasts) used?

161   Response: In fact, we did use endothelial cells, HUVECs cells (human umbilical vein

162   endothelial cells), which are known to be more oxidative-stress and hypoxia sensitive cells

163   than mesenchymal progenitor cells, therefore, if HUVECs are responding appropriately to the

164   manufacturing technique in terms of viability, where hypoxic and free radicals are the main

165   source of possible cell damage, we can argue with more certainly that our methodology is cell

166   friendly or cell compatible. In regard to bone marrow mesenchymal stem cells (BM-MSCs),

167   they have been chosen as the cell source in the cellularized SDVG design for being a less

168   invasive source, easily expanded, immune-evasive allogenic cell type, capable to differentiate

169   to vascular tissues and known to confer positive grafting results in previous vascular graft

170   developments. We have now clarified the rationale behind all cell types used in the

171   fabrication, *in vitro* and *in vivo* studies.

172   The rationale for the selection of HUVECs for the proliferation and LIVE/DEAD assay is now

173   clarified in line 381 ("HUVECs were chosen for this study as they have been identified as more

174   oxidative-stress and hypoxia sensitive cells than progenitor stem cells[50-54], therefore more

175   sensitive   to   free   radical   polymerization   and   hypoxic   conditions   presented   during

176   manufacturing. By performing this assay using HUVECs instead of progenitor cells (e.g. BM-

177   MSCs), masking of the level of compatibility by high resistant cells is avoid, and it can be

178   demonstrated with higher certainly that the biofabrication technique is cytocompatible.")

179   BM-MSCs cells were selected for the fabrication of SDVGs and for the immunomodulatory

180   study in mice, the rationale behind this is now given in line 419 ("On the other hand, bone

181   marrow mesenchymal stem cells (BM-MSCs) are known to have immunomodulatory activity;

182   therefore, it is expected that immunoreaction in presence of this cell type in the SDVG be

183   controlled and the effect of endotoxins ameliorated. It is important to remark that in this

184   study, vascular cell functionality, such as contractility, was not under evaluation, instead

185   functionality of BM-MSCs, known as an excellent cell source for vascular remodeling and

186   regeneration[56] and considered within the SDVG design."), and reinforced in line 587 ("In vivo

187   immunogenicity experiments demonstrate that the encapsulation of BM-MSCs lowers the

188   rejection and inflammation response cause by the subcutaneous implantation of an

189   endotoxin-laden SDVG. The immunosupression cell function is of importance in the design of a

190   new vascular graft, considering a cause of graft failure is associated to chronic inflammatory

191   response[5]") and line 603 ("Additionally, mesenchymal stem cells (MSCs) can be encapsulated

192   as a multipotent source of the biological component with the ability to differentiate to the

193   desired tissues, assist in the recruitment and migration of patient's cells by secreting different

194   chemokines, and help in the formation of a definitive anti-thrombogenic inner layer after

195   implantation[74].").

196   In general terms, vascular cells were not considered in this design due to potential

197   immunorejection that these cells could generate in patients in any future clinical application.

198   In order to use autologous vascular cells, these cells need to be harvested from the same

199   patient. This possibility is considered risky in terms of commercial and clinical viability, as cell

200   harvesting and expansion would require invasive procedures, long-term cell culture and

201   accompanying sterility issues, making the SDVG more expensive and less applicable for urgent

202   situations. iPS on the other hand, are potentially an excellent source for this application due to

203   the low invasiveness of their harvesting procedure, however, reprogramming, expansion and

204   differentiation are still long and expensive procedures for this application, without mentioning

205   the high concern of potential teratoma formation. As identified for use in this study, donated

206   BM-MSCs are considered less invasive, storable and immunotolerated. Additionally, these

207   cells could differentiate, immunomodulate and assist tissue regeneration, being considered as

208   key cells in the process of remodeling and tissue integration for tissue engineered grafts.

209

210

211   Please describe the details of the vessel culture from days 1 to 7.

212   Response: This has been added and detailed in the revised version in the materials and

213   methods section, specifically in the cell culture sub-section in line 819 ("Cell culturing of

214   complete or sectioned SDVGs were performed similarly, except that culture media was

215   additionally supplemented with 1X amphotericin B (15290-026, Gibco, USA). This preventive

216   measure was performed to mitigate exposure to microorganisms post fabrication.").

217

218   Cell survival appears to be equated to function, but it should be made clear that vessel

219   function (e.g. contractility) was not tested in this paper.

220   Response: We agree with this important comment. In the revised version, we have pointed

221   out in the section "Engraftment potential of the encapsulated cells in an immunocompetent

222   mice model" (line 414), more specifically in line 421 ("It is important to remark that in this

223   study, vascular cell functionality, such as contractility, was not under evaluation, instead

224 functionality of BM-MSCs, known as an excellent cell source for immunomodulation and

225 vascular remodeling and regeneration[56] and considered within the SDVG design."), that

226 vascular cell functionality has not been evaluated, and that cell survival and

227 immunomodulatory functionality of encapsulated BM-MSCs has been measured to stablish

228 the cytocompatibility of the manufacturing method. However, we argue that BM-MSC

229 function has been key in the process of remodeling and tissue fusion for other cellularized

230 vascular grafts. We have expanded our discussion in line 589 ("The immunosuppressive cell

231 function is of importance in the design of a new vascular graft, considering a cause of graft

232 failure is associated to chronic inflammatory response[5]. These results prove the importance of

233 fabricating MSC-cellularized grafts to prevent rejection and enhance the functionality of the

234 implant by promoting cell repopulation and definitive tissue remodeling after implantation[74]")

235 and line 603 ("Additionally, mesenchymal stem cells (MSCs) can be encapsulated as a

236 multipotent source of the biological component with the ability to differentiate to the desired

237 tissues, assist in the recruitment and migration of patient's cells by secreting different

238 chemokines, and help in the formation of a definitive anti-thrombogenic inner layer after

239 implantation[74]"), arguing that BM-MSCs functionality could be a key element in the successful

240 grafting of the new SDVG.

241

242 SIVG does not appear to be defined anywhere in the paper

243 Answer: Thank you very much for pointing out this omission. This has been corrected

244 throughout in the revised manuscript.

245

11

246

247

248

249   Reviewer #3 (Remarks to the Author):

250

251   Title: Rapid fabrication of reinforced and cell-laden vascular grafts structurally inspired by

252   human coronary arteries.

253   Authors: Akentjew, T.L., et al.

254

255

256   General Comments:

257   This is an interesting paper describing a combination of spinning and hydrogel technologies,

258   that can deposit concentric layers of cell-laden hydrogels that are interspersed with re-

259   inforcing fibres to provide mechanical strength.

260   Cell layers are contained in a methacryloyl gelatin-alginate (GEAL) substrate, while the

261   reinforcing fibres are made from slow-degrading polycaprolactone (PCL). The idea of

262   interspersing mechanically reinforcing fibres within a living tissue is novel and potentially

263   important. However, this paper has multiple drawbacks that limit enthusiasm substantially.

264   Overall, important information is lacking on the process, and the figures contain many errors

265   and substantially lack clarity. Missing important information includes: what is the diameter of

266   the grafts? How long does the graft manufacturing process take – can cells survive this

267   period? Is the process sterile? How big are the PCL fibres? This is difficult to infer from the

268   paper. What is the phenotype or appearance of the cells in the construct? There is zero

269   histological information. How were MSC harvested and characterized? No information on this

270   either.

271   Also, there is overall a lack of care in preparation of the paper. The figure legends contain

272   many errors, the statistical analysis is uneven and poor overall, and the Methods descriptions

273   do not comport with the data in the paper. Overall, English grammar should be improved as

274   well.

275

276   Response: We thank the reviewer for their helpful comments. We have conducted substantial

277   revisions to address all of the issues pointed out. Specifically, concerning the diameter of the

278   graft, this information was included in line 288 of the main manuscript ("The wall thickness of

279   the SDVG was 0.59 ± 0.17 mm, relatively similar to the combined thickness of the middle and

280   outer graft layer fabricated separately, and the inner diameter 3.6 ± 0.5 mm ").

281   Additionally, in regard to the manufacturing time, this information is now included in line 286

282   ("The complete sterile SDVG fabrication (see material and methods) procedure took an

283   average of 30 min to manufacture once the precursor solutions were prepared "), and

284   discussed in line 617 ("The complete fabrication of an SDVG takes approximately 30 min.

285   Furthermore, this manufacturing equipment has the potential for scale-up to fabricate more

286   than one vascular graft at a time and can be adapted to a self-contained GMP manufacturing

287   unit that can be even utilized in the surgery room or in semi-centralized manufacturing

288   facilities close to healthcare centers. These advantages increase the feasibility of this

289   manufacturing method, lowering cost and manufacturing time, attractive from the

290   perspectives of commercialization and clinical translation.").

291 Regarding the process sterility, this is now clarified in line 738 of materials and methods

292 through a description of precautions taken for sterility maintenance, especially for

293 experiments involving cell culturing or in vivo implantation, quoted here: "The whole

294 manufacturing process was performed under sterile conditions within a biosafety cabinet,

295 including the preparation of methacryloyl gelatin-alginate (GEAL) solution. After chemical

296 modification, and initially during dialysis, the first hour of dialysis is performed using a water

297 solution supplemented with 1% (v/v) chloroform for sterilization[92] . The prepared GEAL

298 mixture was then submitted to 3 cycles of heating and cooling (20 min at 70°C and 20 min at

299 4°C). Additionally, bioburden testing (Inoculation of SDVG extract in Blood agar, Sabouraud

300 Dextrose Agar, Valtek, Chile) and mycoplasma testing (MycoAlertTM Mycoplasma Detection

301 Kit, Lonza) were routinely performed for fabricated SDVG following provider instructions.

302 SDVG constructs used for experiments with cells or animal models were treated with 1% (v/v)

303 penicillin-streptomycin (15140-122, Gibco, USA) in culture media for 24 h.".

304

305 Additionally, the information about dimensions of PCL fibre have been included in line 170

306 ("PCL fibre sublayers fabricated with a target of +/- 21°, had resultant average fibres angles in

307 the cylindrical construct of 31 ± 31° (Fig. 3b) in one orientation and -28 ± 32° (Fig. 3c) in the

308 opposite orientation, with average fiber diameter of 698 ± 253 nm. The PCL fibre sublayer

309 fabricated targeting +/- 67°, exhibited fibre angles of 78 ± 22° (Fig. 3e), while the oppositely

310 oriented fibres were -77 ± 22° (Fig. 3f), with an average fibre diameter of 1.2 ± 0.5 µm").

311 Phenotypic appearance and histological information has been informed now in the live/dead

312 assay of encapsulated cells within different graft sublayer (Fig. 8) and histological analysis of

313   rabbit carotid grafted SDVG, respectively. The last one can be found in the new section

314   "Implantability study in arterial circulation using a rabbit model" in line 502 and Fig. 10.

315   Finally, concerning the harvesting of BM-MSCs, we specify that BM-MSCs were commercially

316   obtained from Lonza, and included the code and lot number in the material and methods

317   section, in the cell culture sub-section, line 810 ("Human umbilical cord endothelial Cells

318   (HUVEC) (ATCC® CRL1730™) and bone marrow derived mesenchymal stem Cells (BM-MSCs,

319   expanded until passage 5) (#PT-2501, Lot# 0000423370, LONZA, USA) were cultured and

320   expanded in high glucose Dulbecco's Modified Eagle's medium (DMEM) (16000-044, Gibco,

321   USA) supplemented with 10%(v/v) fetal bovine serum (FBS) (16000-044, Gibco, USA), 2 mM

322   glutamine (25030-081, Gibco, USA) and 1% (v/v) penicillin-streptomycin (15140-122, Gibco,

323   USA), and incubated at 37°C, 5% CO_2 and 96% of humidity."). Lonza provided a certificate of

324   immunophenotypification and tridifferentiation following the ISSCR recommendations for this

325   type of cells.

326   All figure legends have been corrected, as suggested too by the reviewer Nº 2, and material

327   and methods section improved following the reviewer's suggestions, and addressed one-by-

328   one below. Regarding statistical analysis, more detailed information has been added in

329   material and methods (line 937: "Data are presented as mean ± SD or mean ± SEM, also

330   indicated in the figure caption. Statistical significance was determined using two-tailed Mann-

331   Whitney U test in compliance and cell density of bio-inspired SDVGs (Table 1, and Fig 8). Two-

332   tailed Mann-Whitney U test was also applied for the in vivo immunosuppression functional

333   experiments in the mouse model, platelet activation assay and clotting time assay (Fig 9, 10,

334   respectively). All statistically analyzed data comply with the non-parametric Mann-Whitney U

335   test. Platelet activation assay was performed utilizing 3 human blood samples from different

15

336 donors and conducted in experimental triplicates, whereas clotting time assay was done using

337 4 human blood donors in experimental triplicates. Sample size calculations were determine

338 using   the   on-line   resource   from   IACUC   of   Boston   University

339 (https://www.bu.edu/researchsupport/compliance/animal-care/working-with-

340 animals/research/sample-sizecalculations-iacuc/) [98,99], with a power level of 95% and

341 estimated variance from previous publications. Specifically for compliance experiments,

342 estimated variance was determined based on Claes, E.[45] and van Andel, C. J. et al.[46], whereas

343 for the immunocompetent mice model was based on Campos-Mora, M. et al.[96]. All compared

344 groups are considered to have similar data distribution. In the implantability study in arterial

345 circulation using a rabbit model, results were presented as preliminary study, therefore only 2

346 animals per group were performed and no statistical analysis presented.

347 All experimental outcomes were obtained after blinded quantitative analysis of samples

348 results. For all statistically analyzed experiments, 95% of confidence was used and significance

349 was denoted as *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$. "n" values for each experiment are

350 informed in the figure caption."), including as well the "n" values for each experiment and

351 informed in the figure caption.

352

353 Specific Comments:

354 1. Line 123: the statement is made that the GEAL solution is pre-crosslinked, but then the

355 authors state that the dipped layer is exposed to UV light to crosslink the material. please

356 clarify.

357 Response: We thank the reviewer for pointing this out. The GEAL solution was not pre-

358 crosslinked, we have now modified the body text to clarify this and revised Figure 2 to include

359 the crosslinking/photo-crosslinking step within the vessel production sequence. We have

360 clarified this in the manuscript in line 143: "Subsequently, the rod containing the orientated

361 PCL fibre sublayers was immersed (dipped) into the GEAL solution and slowly retracted whilst

362 spinning to allow homogenous GEAL layer photo-crosslinking using a lateral UV source (Fig.

363 2b)."

364

365 2. The phrase "circumferential axis" is used throughout the paper and is unclear to this

366 reviewer.

367 Response: This has now been clarified in the revised manuscript. Figure 1 has been revised

368 with a scheme of the multilayer vascular graft, defining the circumferential axis for clarity.

369

370 3. After spraying the PCL layers, do the fibres fuse at the points of contact? What is the data

371 for or against fibre fusion, which will significantly impact the vessel mechanical properties?

372 What is the fibre diameter, is it uniform, how is it controlled?

373 Response: We thank the reviewer for pointing this out. We have included more detail

374 concerning fibre morphologies, diameters and confirmed that the fibres are individualized by

375 SEM and CT investigation (Fig. 4 and 6), however, some level of fusion between fibres cannot

376 be discarded. We have expanded the text concerning the fibres and the implication of their

377 fusion on mechanical properties. We have included those comments in line 295 ("The fibres

378 are individualized, with minimal fibre fusion evident, according to SEM and CT (Fig. 4d and 6c,

17

379   repectively). Fibres fusion is an aspect of concern in the design of the SDVG, because fibres

380   fusion would indeed impact the mechanical response, mainly due to force distribution

381   amongst fibres at fixed contact points. Overall in the actual SDVG, free displacement of fibres

382   would be possible during stretching and recoil. ”). Fusion of the fibres can be controlled by

383   fixing the distance between the SBS head (fibre emitting) and collection, if solvent evaporation

384   is insufficient prior to hitting the target then some fusing of the fibres can occur; we specified

385   the collection distance used here to obviate fibre fusion. Concerning fibre diameter, values

386   fluctuate between 500 nm and 1800 nm approximately depending on the angular orientation

387   of the SBS respect to the circumferential axis of the construct or rod. Information about the

388   average values and standard deviation was included in line 170 (”PCL fibre sublayers

389   fabricated with a target of +/- 21°, had resultant average fibres angles in the cylindrical

390   construct of 31 ± 31° (Fig. 3b) in one orientation and -28 ± 32° (Fig. 3c) in the opposite

391   orientation, with average fiber diameter of 698 ± 253 nm. The PCL fibre sublayer fabricated

392   targeting +/- 67°, exhibited fibre angles of 78 ± 22° (Fig. 3e), while the oppositely oriented

393   fibres were -77 ± 22° (Fig. 3f), with an average fibre diameter of 1.2 ± 0.5 µm.”). Although we

394   have not explored in this work the variable controlling the fibre diameters during

395   manufacturing, except for deposition angle (see lines 174-181), it is known from previous

396   research in SBS and electrospinning that solvent type and mixture and polymer concentration

397   are typical variable by which diameter can be controlled.

398

399   4. The legend for Figure 4 is very confusing – panels a-c are stated as having data in the grey

400   lines, but these lines are identical across the images, and the dotted lines are not described at

401 all. It is pretty impossible to tell what this figure is saying. Do the authors have data to show

402 on actual stress-strain curves of native human coronary arteries?

403 Response: We are very thankful for the reviewer comments and get feedback concerning

404 improvements of the figures. We have substantially revised all the figures in the manuscript

405 for clarity, explaining in more details the data described by the plotted lines. The legend has

406 been corrected too and clarified that the data of native human coronary were originated in

407 previous published studies.

408 Specifically, concerning the caption of Fig. 4, we have added the modifications and the new

409 caption resulted as follow: "Figure 4: **Iterative improvement towards a J-shape stress-strain**

410 **curve combining wavy fibre deposition and preconditioning:** (a) Optical microscope image of

411 a PCL fibre sublayer fabricated at a deposition angle of 21° after 1 cycle of fibre deposition

412 with continuous clockwise rod spinning. (b) Optical microscope image of a PCL fibre sublayer

413 fabricated at a deposition angle of 21° after 1 cycle of fibre deposition with alternated rod

414 spinning and after the preconditioning step. (c) Scanning electron microscopy image of a

415 series of 24 PCL fibre sublayers fabricated at a deposition angle of +/-67°. (d) Scanning

416 electron microscopy image of a series of 24 PCL fibre sublayers fabricated at a deposition

417 angle of +/-67° with alternated rod spinning and after the preconditioning step. (e)

418 Longitudinal strain-stress curve of a series of 24 PCL fibre sublayers fabricated at a deposition

419 angle of +/-21°, with and without a stretch preconditioning step of 5 cycles of

420 loading/unloading at 30% strain and wavy fibre deposition using the alternating rod spinning

421 during angled fibre deposition. The grey line represents previously published stress-strain

422 mechanical behavior of the media layer of the human coronary artery under longitudinal

423 tensile testing[15]."

As specified in the modified caption, human coronary data was obtained from a previous work (Holzapfel et al (2005)), in which 13 donated human coronary arteries were evaluated. From this data a constitutive mathematical model capable to describe the mechanical behavior in uniaxial tensile testing was obtained. In our work, mechanical tensile testing was applied in the same manner as their study, including strain rate as further detail in our response to referee 2.

5. Similarly the legend for Figure 5 makes no sense – the grey lines and solid squares that are talked about the in the legend simply aren't there in the figure. And, panels b-e contain multiple black lines of unclear significance- if these are replicates, then should not some error bars be included or something? In Figures 5 and 7, where is the "grey region of the range of the model of the mechanical properties" derived from? This is not at all clear to the reader, and serves only to confuse.

Response: All figures have been reworked, as addressed in our responses to reviewer Nº 2. Specifically concerning this reviewer's comments for Fig 5 and 7: Black lines were replicates for the fabricated SDVG, which are expressed now as averages with their corresponding standard deviation. The grey zone (now light green zone) corresponds to the range of values obtained for native coronary arteries, which were extracted from previous work (Holzapfel et al (2005)). In our work, mechanical tensile testing was applied in the same manner as this study (Holzapfel et al (2005)), including pre-conditioning and strain rate. This was clarified in the caption and text. Additionally, legends were included in the figures to clarify the designation of colours and symbols. In figure 5, caption was modified and improved the clarity:

446 "Figure 5: **Stress-strain curves of the outer and middle graft layers based on GEAL reinforced**

447 **PCL sublayers.** a) Iterative improvement of the middle and outer graft layers: longitudinal

448 tensile testing of the middle and outer graft layers using different numbers of middle and

449 outer graft sublayers in the construct. Stress-strain curves of the outer graft layer consisting of

450 different layer numbers. Grey dotted lines represent the average longitudinal stress-strain

451 curve of the native adventitia (closed circles) and media (closed diamonds) layer of human

452 coronary arteries[15]. b) and c) Longitudinal and circumferential stress-strain curve of the outer

453 graft layer composed of 5 graft (GEAL/PCL) sublayers. Green dashed lines in b and c represent

454 the average longitudinal and circumferential stress-strain curves of the native media layer of

455 coronary arteries[15], respectively. d) and e) Longitudinal and circumferential stress-strain curve

456 of the middle graft layer composed of 4 graft (GEAL/PCL) sublayers. The green dashed lines

457 in d and e represent the average longitudinal and circumferential stress-strain curve of the

458 native adventitia layer of human coronary arteries[15] respectively. (n=3). The shaded green

459 zones in the figures represent the range of results obtained for native human coronary

460 arteries[15]. Error bars = standard deviation, (n=3 independent experiments)."

461 Concerning figure 7 caption was modified too and improved clarity:

462 "Figure 7: Mechanical evaluation of the fabricated SDVG. a-b) Stress-strain curves of the SDVG

463 (black line) and the human coronary artery in a) longitudinal and b) circumferential stretching

464 directions (n = 5 independent experiments). The green dashed lines in a and b represent the

465 longitudinal and circumferential stress-strain curve of native human coronary arteries[46]. The

466 green shaded zones represent the range of results obtained for native human coronary

467 arteries[46] . (c) Cyclic tensile testing in the circumferential direction. d-f) Profiles of diameter

468 change ratio (D/D0) as function of pressure applied to the SDVG (black line, n = 5 independent

469 experiments) compared with human coronary arteries (green dashed lines, n = 5 independent

470 experiments)[45,46] at three different values of axial pre-stretch during testing (ez). d) ez=10% of

471 axial pre-stretch.e) ez=20% of axial pre-stretch. f) ez =25% of axial pre-stretch. Error bars =

472 standard deviation."

473

474

475

476 6. In Table 1, what is the "n" value for these data? This bears on the statistical significance and

477 differences.

478 Response: We thank the reviewer for pointing this out. This has now been corrected in Table 1

479 of the revised manuscript, including details as to how the data was obtained. Specifically in

480 table 1 caption the following was included: "Table 1: Bio-inspired small diameter vascular graft

481 (n=5) and human coronary artery (n=5, data obtained from Claes, E.[45] and van Andel, C. J. et

482 al.[46] compliance (%C) (10-2 mmHg) at different pressure ranges and longitudinal pre-stretch

483 during testing (ez). Standard deviation is presented too (±). ".

484

485 7. Figure 8 needs scale bars. What is the meaning of the blue color – does this signal live or

486 dead cells or proliferating cells? What are the dimensions of the construct? The reagent used

487 does not show cell replication per se – it appears to show mitochondrial activity??

488 Response: We have revised the manuscript and re-worked Figure 8 to include a carefully

489 annotated description in the caption of Figure 8. The dimensions of the graft are now clarified

490   in line 286 ("The complete sterile SDVG fabrication (see material and methods) procedure

491   took an average of 30 min to manufacture once the precursor solutions were prepared. The

492   wall thickness of the SDVG was 0.59 ± 0.17 mm, relatively similar to the combined thickness of

493   the middle and outer graft layer fabricated separately, and the inner diameter 3.6 ± 0.5 mm.

494   ."). Scale bars were included for the SDVG fluorescent image of SDVG in figure 8.

495   Additionally, the reviewer's concern about proliferation assay and mitochondrial activity has

496   been clarified in the manuscript in line 380 ("In order to evaluate the cell viability after

497   manufacturing, a cell proliferation assay based on mitochondrial activity was performed post-

498   fabrication."), and line 390 ("Nevertheless, limited diffusion of nutrients and reagents of the

499   proliferation kit within the graft must be taken into consideration; this could lead to an

500   underestimation of the mitochondrial activity, hence cell survival and proliferation").

501

502   8. Figure 9 shows cells counts from histology, but there are no histological images shown in

503   the main paper. This is very disappointing.

504   Response: We thank the reviewer for pointing this out. Histological images of rabbit carotid

505   grafted SDVG are presented now in section "Implantability study in arterial circulation using a

506   rabbit model" (line 502) and supporting information (Fig. S8). These images correspond to

507   H&E staining of cellularized SDVG after 14 and 30 days post-implantation (Fig. 10h,i), and H&E

508   staining of acellularized (Fig S8a) and cellularized SDVG (Fig S8b) after 30 days post-

509   implantation. On the other hand, cell counting presented in figure 9, corresponds to cells

510   obtained from graft-draining lymph nodes to evaluate the level of immunoreaction. This has

511   been clarified in the caption and as well in line 425 ("Analysis of immunocompetent mice with

512   dorsal subcutaneous implantations demonstrate that SDVGs with encapsulated endotoxins

513   and without BM-MSCs induced graft rejection when implanted subcutaneously, characterized

514   by a lack of graft incision healing (see Fig. S7a), an increased number of total cells isolated

515   from graft-draining lymph nodes (dLNs) (mouse axillary and brachial lymph nodes), and an

516   increased percentage of CD4+ memory T cells and B cells in dLNs compared to BM-MSCs

517   cellularized graft (Fig. 9 b, c, and d respectively) "

518

519   9. In the Methods section, the Histology paragraph talks about skin transplants and positive

520   controls for immunity, and talks about FACS analysis using different markers for immune cells,

521   but none of this data is shown in the paper. It appears as if this text was taken from another

522   manuscript, since it does not match up with the data in this paper?

523   Response: We thank the reviewer for pointing this out. This mistake has been corrected in

524   substantially revised materials and methods section, and as well further clarified in the

525   caption of Figure 9. Concerning the Figure 9, the following underlined modifications has been

526   included:

527   "Figure 9. Descriptions of immune results. (a) Schematic overview of the experimental

528   immune challenge to assess the immunomodulatory function of laden BM-MSC in the SDVG.

529   (b) Number of cells in dLN. (c) Frequency of CD62L- CD44+ cells within CD3+ CD4+ cell

530   population, corresponding to memory T Cells. (d) Frequency of CD19+ cells in dLN,

531   corresponding to B cells. (e) Frequency of CD25+ cells within CD3+ CD4+ cell population,

532   corresponding to activated T cells. (f) Frequency of CD62L+ CD44- cells within CD3+ CD4+ cell

533   population, corresponding to naïve T Cells. (g) Frequency of CD25high Foxp3+ cells within

24

534   CD3+ CD4+ cell population, corresponding to regulatory T cells. Naïve = non-operated control

535   mice; Suture control = operated mice without any graft; Allogeneic = operated mice with

536   allogenic skin graft. Error bars = standard error of the mean. * $p<0.05$; ** $p<0.01$; ns: non-

537   significant. n=5 animals with 5 different SDVG fabrications. Statistical analysis was conducted

538   using the Mann–Whitney U test."

539

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors are commended for performing additional SVDG strength characterization and a rabbit carotid artery interpositional implantation study. The reported strength values are reasonable albeit lower than for other TEVG that have attained large animal testing. Unfortunately, acute clotting of the grafts in the rabbit model indicates the graft, despite its noteworthy structure and compliance properties, is not yet a successful TEVG in terms of a large animal implantation; thus, the study, while comprehensive and well presented, is of limited significance given the state of the TEVG field, and I do not think its probable impact merits publication in Nature Communications.

Reviewer #2 (Remarks to the Author):

This is an exciting paper describing an innovative approach for fabrication of vascular grafts that effectively mimic the mechanical response of native vessels and provide a scaffold for vascular cells to function and remodel. The authors have effectively responded to the reviewers' recommendations, and the manuscript is substantially improved, particularly with inclusion of additional results addressing graft strength and performance in a rabbit model. There are still some grammatical issues here and there, but they are not so extensive as to lead to confusion.

One minor suggestion: Please define (in the figure caption) the angle alpha shown in Figure 1. Is it the parameter associated with the angles referenced in the caption for Fig. 1?

Ken Monson

Reviewer #3 (Remarks to the Author):

Title: Rapid fabrication of reinforced and cell-laden vascular grafts structurally inspired by human coronary arteries.

Authors: Akentjew, T.L., et al.

General Comments: This is a substantially revised manuscript regarding the use of electrospinning techniques to create PCL-gel composite tissues with mechanics similar to native artery. The changes in response to the previous review are extensive, and so this reads almost like a new paper.

Compared to the prior submission, the clarity of the procedures and the characterization of the scaffolds, particularly the mechanical characterization, are vastly improved. This reviewer is again impressed at the extent to which a biological approach to orientation of PCL fibers led to expected and predicted compliance properties. In addition, the mechanical properties of these small-diameter conduits do now appear to be compatible with arterial implantation. As such, this material characterization and the methodology combine to make this an important advance for research in the area of synthetic arterial grafts.

But while there are many improvements, there are some new weaknesses in the paper, mostly pertaining to the biological characterization of the materials. To remedy some of these deficiencies, it would be suitable (in this reviewer's opinion) to simply remove some of the confusing biological data, since it does not add significantly to the important parts of this story as it currently stands. In addition, the paper is now QUITE LONG, and should be streamlined to about half of its length, in terms of text. Specific comments below.

Specific Comments:

1. In Figure 5, providing more explicit labels on the y-axes would help readers understand which wall stress they were looking at.

2. Figure 6 does not add a lot to the paper, and could be omitted or moved to a supplement.

3. Figure 7 – again, more explicit y-axis labels, with directionality for panels a-c, and with amount of pre-stretch for panels d-f.

4. Table 1 could be omitted, as not adding a lot to the figures already presented.

5. English language correction, line 396, should read: "highly resistant cells is avoided"

6. Regarding results in lines 394-406, it should be noted that 0.02 – 0.2% survival is still very poor for HUVEC in the construct. Is poor survival the reason that HUVEC were abandoned for later implantations? Also, what was the survival of BM-MSC when implanted into the constructs?

7. The authors seem to have an incomplete appreciation of the effects of endotoxin. In lines 430-444, there is discussion of endotoxin, MSC, rejection, etc. Some corrective observations:

a. HUVEC will be susceptible to endotoxin – it is toxic to endothelium

b. Endotoxin is a strong inducer of inflammation, but not of adaptive immunity, per se. Therefore, the B- and T-cells that migrated to the implant were likely part of a non-specific inflammatory response, rather than part of an actual rejection event. If the implants did not have cells, then rejection, in the proper sense, could not have occurred.

c. Adding MSC to this cocktail may have resulted in fewer cells on FACS, but the meaning of this observation is really not clear.

d. This reviewer would recommend that the cutaneous implants be struck from the paper, since they do not add value in terms of understanding of the construct, and provide some confusing information.

8. For the rabbit carotid implants, it is worthwhile to point out that endotoxin itself can induce endothelial inflammation and hence thrombosis. This may have been why all of the implanted grafts clotted within a short time period. The amount of endotoxin in the constructs should be quantified to gain a better understanding of what is going on here.

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors are commended for performing additional SVDG strength characterization and a rabbit carotid artery interpositional implantation study. The reported strength values are reasonable albeit lower than for other TEVG that have attained large animal testing. Unfortunately, acute clotting of the grafts in the rabbit model indicates the graft, despite its noteworthy structure and compliance properties, is not yet a successful TEVG in terms of a large animal implantation; thus, the study, while comprehensive and well presented, is of limited significance given the state of the TEVG field, and I do not think its probable impact merits publication in Nature Communications.

Response: We thank the reviewer for their positive comments regarding our additional investigation to strength characterization and the rabbit carotid study.

Although we understand the author's concern pointing to the fact that the paper is not showing a successful long-term patency in a large animal model, the main focus of the manuscript is the new technology based on an innovative combination of modular and automated technologies capable to produce a tissue engineered product with standardized features, which are broadly considered essential for the efficacy of a vascular graft. We present results that show good potential towards clinical usage, specifically, platelet activation, coagulation, burst pressure, suture strength and hemostasis. Additionally, the main manuscript argues at the end of the discussion section about possible reasons that could cause the thrombus formation, basically indicating that a carotid rabbit model is not optimal for evaluating long-term patency especially for an engineered graft with mechanics and wall sizes corresponding to human coronary arteries. Therefore, it is mentioned too that only in a large animal models, the *in vivo* relevance of mechanical matching and the graft design will be fully demonstrated, which is part of our ongoing research, moving beyond the scope of the current manuscript. In regard to the possible reasons that caused the thrombus formation after 12 h, we included in the previous revision that luminal unalignment of the SDVG and the native rabbit carotid at the anastomosis zone could cause blood recirculation and stagnation, therefore, coagulation (see lines 554-556 in the new revision). In this new version, we have additionally included a second possible cause, which refers to inflammation-induced thrombogenesis due to the endotoxin levels of the SDVG (see lines 558-566 in the new revision). To complement the discussion, we quantified and reported the endotoxin level in fabricated SDVG and performed in vivo immunogenicity assay to evaluate the immune reaction. These results were included in the new manuscript and new supporting information, respectively, and

concluded that inflammation cannot be discarded as a possible cause of thrombus formation in the present study.

The technology presented in this manuscript is a unique rapid manufacturing process that integrate the use of natural biomaterial and cells simultaneously, conforming a disruptive strategy to overcome standardization, manufacturing control, regulatory and commercial hurdles present in the tissue engineered field, which in combination could reduce the gap for this type of product to commercialization and impact in the public health.

Reviewer #2 (Remarks to the Author):

This is an exciting paper describing an innovative approach for fabrication of vascular grafts that effectively mimic the mechanical response of native vessels and provide a scaffold for vascular cells to function and remodel. The authors have effectively responded to the reviewers' recommendations, and the manuscript is substantially improved, particularly with inclusion of additional results addressing graft strength and performance in a rabbit model. There are still some grammatical issues here and there, but they are not so extensive as to lead to confusion.

One minor suggestion: Please define (in the figure caption) the angle alpha shown in Figure 1. Is it the parameter associated with the angles referenced in the caption for Fig. 1?

Response: We thank the reviewer for his comments. We have revised the manuscript for grammatical and English errors throughout.

Additionally, we have included a definition for the angle alpha in the revised caption of figure 1, that effectively correspond to the deposition angle as stated by the reviewer.

"Figure 1: Scheme composition of the middle and outer graft layers. The middle graft layer comprises a series of four PCL/GEAL sublayers, hereafter called middle graft sublayers, with fibres deposited at angles of +/- 21° and GEAL sublayer generated after two cycles of dipping and photo-crosslinking. The outer graft layer is composed of a series of five PCL/GEAL bilayers, hereafter termed outer graft sublayers, with fibres deposited at angles of +/- 67° and GEAL sublayer generated after three cycles of dipping and photo-crosslinking. The deposition angle is represented by "α".

Reviewer #3 (Remarks to the Author):

Title: Rapid fabrication of reinforced and cell-laden vascular grafts structurally inspired by human coronary arteries.

Authors: Akentjew, T.L., et al.

General Comments: This is a substantially revised manuscript regarding the use of electrospinning techniques to create PCL-gel composite tissues with mechanics similar to native artery. The changes in response to the previous review are extensive, and so this reads almost like a new paper.

Compared to the prior submission, the clarity of the procedures and the characterization of the scaffolds, particularly the mechanical characterization, are vastly improved. This reviewer is again impressed at the extent to which a biological approach to orientation of PCL fibers led to expected and predicted compliance properties. In addition, the mechanical properties of these small-diameter conduits do now appear to be compatible with arterial implantation. As such, this material characterization and the methodology combine to make this an important advance for research in the area of synthetic arterial grafts.

But while there are many improvements, there are some new weaknesses in the paper, mostly pertaining to the biological characterization of the materials. To remedy some of these deficiencies, it would be suitable (in this reviewer's opinion) to simply remove some of the confusing biological data, since it does not add significantly to the important parts of this story as it currently stands. In addition, the paper is now QUITE LONG, and should be streamlined to about half of its length, in terms of text. Specific comments below.

Specific Comments:
1. In Figure 5, providing more explicit labels on the y-axes would help readers understand which wall stress they were looking at.
2. Figure 6 does not add a lot to the paper, and could be omitted or moved to a supplement.
3. Figure 7 – again, more explicit y-axis labels, with directionality for panels a-c, and with amount of pre-stretch for panels d-f.
4. Table 1 could be omitted, as not adding a lot to the figures already presented.

5. English language correction, line 396, should read: "highly resistant cells is avoided"

6. Regarding results in lines 394-406, it should be noted that 0.02 – 0.2% survival is still very poor for HUVEC in the construct. Is poor survival the reason that HUVEC were abandoned for later implantations? Also, what was the survival of BM-MSC when implanted into the constructs?

7. The authors seem to have an incomplete appreciation of the effects of endotoxin. In lines 430-444, there is discussion of endotoxin, MSC, rejection, etc. Some corrective observations:

a. HUVEC will be susceptible to endotoxin – it is toxic to endothelium

b. Endotoxin is a strong inducer of inflammation, but not of adaptive immunity, per se. Therefore, the B- and T-cells that migrated to the implant were likely part of a non-specific inflammatory response, rather than part of an actual rejection event. If the implants did not have cells, then rejection, in the proper sense, could not have occurred.

c. Adding MSC to this cocktail may have resulted in fewer cells on FACS, but the meaning of this observation is really not clear.

d. This reviewer would recommend that the cutaneous implants be struck from the paper, since they do not add value in terms of understanding of the construct, and provide some confusing information.

8. For the rabbit carotid implants, it is worthwhile to point out that endotoxin itself can induce endothelial inflammation and hence thrombosis. This may have been why all of the implanted grafts clotted within a short time period. The amount of endotoxin in the constructs should be quantified to gain a better understanding of what is going on here.

Response: We thank the reviewer for their helpful comments. As suggested by the reviewer, we have moved some biological data from the main manuscript and included in the supporting information (see below for further details). Additionally, we have clarified and simplified the information obtained from the immunogenicity assays and presented in the main manuscript (complete results are included now in the supporting information). Also, we have shortened the text in about 1500 words to adjust to the length of other tissue engineering related papers previously published in Nature Communication and performed new experiment to tackle the endotoxin issue in this work. Following the specific comments, we have undertaken the following modification, addressed here comment by comment (reviewer #3 comments in blue):

Reviewer #3 comment 1. In Figure 5, providing more explicit labels on the y-axes would help readers understand which wall stress they were looking at.

- Following the reviwer's suggestions, a schematic representation of the direction of tensile testing was included in our now revised figures 3, 4, 5 and 6 for clarity.

Reviewer #3 comment 2. Figure 6 does not add a lot to the paper, and could be omitted or moved to a supplement.

- Figure 6 of the previous submitted manuscript was removed from the main manuscript as suggested by the reviewer and is now included in supporting information.

Reviewer #3 comment 3. Figure 7 – again, more explicit y-axis labels, with directionality for panels a-c, and with amount of pre-stretch for panels d-f.

- Please see our response to reviewer #3 in comment 2.

Reviewer #3 comment 4. Table 1 could be omitted, as not adding a lot to the figures already presented.

- Following the reviewer´s suggestion, Table 1 has been removed and is now included in the supporting information.

Reviewer #3 comment 5. English language correction, line 396, should read: "highly resistant cells is avoided".

- After shortening the main manuscript, line 396 has been removed, therefore, the specific language correction was not necessary.

Reviewer #3 comment 6. Regarding results in lines 394-406, it should be noted that 0.02 – 0.2% survival is still very poor for HUVEC in the construct. Is poor survival the reason that HUVEC were abandoned for later implantations? Also, what was the survival of BM-MSC when implanted into the constructs?

- Although we have removed this part from the newly revised manuscript, the following discussion is necessary to be raised in order to clarify the reviewer´s concern. In the previous submitted manuscript, we have attributed the low signal of metabolic activity to the limited diffusion of reagents of the proliferation kit within the graft; this could lead to an underestimation of the mitochondrial activity of cells within the graft. This is particularly true for encapsulated cells within hydrogels with limited solvent access. However, we cannot discard that the lack or decrease in the capacity of WST-1 reduction of cells is derived either from a metabolic resting induced by a free radical-derived oxidative stress during photo-crosslinking (1. Cell Cycle. 2015 Jul 3; 14(13): 2022–2032 2. Ann Biomed Eng. 2017 Feb; 45(2): 360–377. 3. Gene 337 (2004) 1 – 13), or by NADH depletion in response to higher concentration of free radicals and hypoxia after photo-crosslinking (1. FASEB J. 2009 Sep; 23(9): 3159–3170. 2. Free Radic Biol Med.

2015 Feb; 0: 281–291), or simply by an interference of the photoinitiator-derived free radical with the reduced form of the 1-Methoxy-5-methylphenazinium methyl sulfate during the electron transfer in the reduction process of WST-1 to formazan. Although these phenomena could occur in conjunction and affect the reduction of WST-1 during the assay, viability measurement based on assays that evaluate membrane integrity still indicate that cells are viable (please see Figure 7 of the new manuscript). Considering the mentioned doubts about the proficiency of this method in measuring cell viability in the actual cell-encapsulation scenario, and the fact that the cell viability and compatibility of cells within GelMA hydrogels has been previously explored by this group (Biofabrication. 2016 Dec 1;9(1):015001) and other research groups (Biomaterials. 2010 Jul;31(21):5536-44), we have decided to include only live/dead assay and immunosuppression functionality in the *in vivo* model as proofs of viability of cells in the present manuscript.

Concerning the decision of abandoning HUVECs in the final SDVG design, this is not related to the survival of HUVECs but to the expected and more appropriate role of BM-MSCs in the final design with potential regenerative activity after implantation. Reasonings in this regard are included in the new revision:

In lines 363-383:
"Having considered the incorporation of a cellular component essential for the design and successful outcome in a transplantation scenario[32,49,50], important practical implication must be taken into account when choosing an appropriate cell type. Although autologous vascular cells are the preferable choice, invasive harvesting and long-term culturing, specially for elderly patients, make this option risky in terms of commercial and clinical viability. Induced pluripotent stem cells on the other hand, are potentially an excellent source for this application[51] due to the low invasiveness of their harvesting procedure and autologous nature, however, reprogramming, expansion and differentiation are still a long and expensive procedures, and the frequency of point mutations[52] has generated serious concern about the safety of these cells. Allogenic bone marrow mesenchymal stem cells (BM-MSCs) are considered less invasive, storable, economically feasible, immunotolerated, and have been physiologically implicated in vascular repair and remodeling[53]. Additionally, BM-MSCs are known to have immunomodulatory activity; therefore, it is expected that immunoreaction or inflammation in presence of this cell type in the SDVG would be controlled or ameliorated. In this regard, BM-MSCs has been proposed in this study as a source of biological function for the actual SDVG design. Analysis of

immunocompetent mice with dorsal subcutaneous implantations has demonstrated that SDVGs with encapsulated BM-MSCs are capable to control an inflammatory response, whereas non-cellularized SDVG not (see Fig. S9, S10 and supporting information for further details). This demonstrates also that cells maintain their viability and functionality after being subjected to the manufacturing process. It is important to remark that in this study, vascular cell functionality, such as contractility, was not under evaluation, instead functionality of BM-MSCs, known as an excellent cell source for immunomodulation, vascular remodeling and regeneration[54]".

In regard to the BM-MSCs survival, quantification of live cells was included in the new manuscript in lines 340-344: "Using this time bone marrow-derived mesenchymal stem cells (BM-MSCs), and a LIVE/DEAD® cell staining assay, viability analysis of cells located within different sublayers of the fabricated SDVG was performed at different time points of static cell culturing (Fig. 7f-h). Evaluation on day 7, 14, 21 and 28 resulted in 71, 84, 87 and 92% viability respectively (Fig. S8). These results confirm cells survival and even proliferative capacity on day 28 post fabrication (Fig 7i)".

Reviewer #3 comment 7. The authors seem to have an incomplete appreciation of the effects of endotoxin. In lines 430-444, there is discussion of endotoxin, MSC, rejection, etc. Some corrective observations:

Considering the reviewer`s suggestion, the subcutaneous implantation was moved to the supporting information and taken only as an additional information to prove the immunosuppressive capacity of BM-MSCs in the present construct. Additionally, the used of a low endotoxin alginate for the SDVG construct was specified in the materials and methods section in lines 593-594 and 600-602, clarifying too that the use of an alginate with higher level of endotoxin was only for the subcutaneous assay in the context of testing the immunosuppressive activity of encapsulated BM-MSCs.

Reviewer #3 comment 7a. HUVEC will be susceptible to endotoxin – it is toxic to endothelium.

Comment 7a: Although the LPS toxicity on endothelial cells has been previously stablished by other authors (Infect Immun. 1993 Aug; 61(8): 3149–3156.), in this investigation, low endotoxin alginate has been used in the fabrication of SDVG, as mentioned previously, therefore, the authors of this article did not consider the possible toxicity of endothelial cells after the SDVG implantation. However, discussion and experimental proofs regarding this point are mentioned in the paper and in this document further below.

As well mentioned by the reviewer, and due to the nature of the implanted acellular SDVG, effectively, we should not talk about rejection, therefore, we have changed this concept for inflammation and wound healing, when it comes to consider evaluations of the faster incision closure for cellularized SDVG. These changes are listed below:

- o In lines 375-382 of the main manuscript: "Analysis of immunocompetent mice with dorsal subcutaneous implantations has demonstrated that SDVGs with encapsulated BM-MSCs are capable to control an inflammatory response, whereas non-cellularized SDVG not (see Fig. S9, S10 and supporting information for further details). This demonstrates also that cells maintain their viability and functionality after being subjected to the manufacturing process. It is important to remark that in this study, vascular cell functionality, such as contractility, was not under evaluation, instead functionality of BM-MSCs, known as an excellent cell source for immunomodulation, vascular remodeling and regeneration[54]."

- o In Supporting information, subsection "Results of immunosupressive activity of the encapsulated cells in the SDVG", the following has been included: "Analysis of immunocompetent mice with dorsal subcutaneous implantations demonstrate that SDVGs with encapsulated endotoxins and without BM-MSCs induced a graft-derived inflammatory reaction, characterized for instance by a delayed graft incision healing (see Fig. S9a), an increased number of total cells isolated from graft-draining lymph nodes (dLNs) (mouse axillary and brachial lymph nodes), and an increased percentage of CD4+ memory T cells and B cells in dLNs compared to BM-MSCs cellularized graft (Fig. S10 b, c, and d respectively). Whereas subcutaneously implanted SDVGs with encapsulated BM-MSCs, exhibited no signs of inflammation and the incision healed after 14 days (Fig. S9b). Reduced cell numbers in dLNs, and an augmented percentage of activated CD4+ T cells, CD4+ naïve T cells, and CD4+ regulatory T cells (Fig. S10 e, f and g respectively) compared to implanted acellularized grafts was also found. These results, and considering the immunophenotypic data of allogeneic skin graft, known for conducting inflammation and immune rejection[7,8],

suggests that an immunomodulation is being carried out by the viable and functional encapsulated BM-MSCs, mainly characterized by an immunotolerance of the endotoxin-laden graft[9], decreased presence of B-cells[10] and increased presence of regulatory T cells in dNLs[11], all previously described as functions of BM-MSCs."

In regard to the increase of T-cells and B-cells induced by the presence of endotoxin in the subcutaneously implanted SDVG, we agree with the reviewer about the non-specific inflammatory response as the main cause of increased number of immune cells. In the succession of event after incision, it has been reported that within the first week, recruitment of antigen-presenting cells, T-cells (J Immunol. 2010 May 15; 184(10): 5423–5428.) and B-cells (Wound Repair Regen. 2017 Sep; 25(5): 774–791) may occur as a non-specific respond. However, LPS is considered a strong adjuvant with implication in clonal expansion of T-cells for example (Crit Rev Immunol. 2008; 28(4): 281–299), either derived from presentation of SDVG biomaterial antigens or unknown antigen expressed on damaged keratinocytes (J Immunol. 2010 May 15; 184(10): 5423–5428.). Nevertheless, these considerations are included in the new manuscript, describing the phenomenon more as a non-specific inflammatory reaction and not as an implant rejection.

Reviewer #3 comment 7c. Adding MSC to this cocktail may have resulted in fewer cells on FACS, but the meaning of this observation is really not clear.

Although we have move these results and discussion to supporting information, we have included an explanation in supporting information referring to the immunomodulatory effect of BM-MSCs in the inflammatory reaction triggered by the implantation of endotoxin-laden SDVG:

"…….suggests that an immunomodulation is being carried out by the viable and functional encapsulated BM-MSCs, mainly characterized by an immunotolerance of the endotoxin-laden graft[6], decrease in the number of dLN cells, decreased presence of B-cells[7] and increased presence of regulatory T cells in dNLs[8], all previously described as functions of BM-MSCs…..".

Additionally, we have simplified the message of these results in the main manuscript in order not to deviate the focus of the study:

In lines 375-382: "Analysis of immunocompetent mice with dorsal subcutaneous implantations has demonstrated that SDVGs with encapsulated BM-MSCs are capable to control an inflammatory response, whereas non-cellularized SDVG not (see Fig. S9, S10 and supporting information for further details). This demonstrates

also that cells maintain their viability and functionality after being subjected to the manufacturing process. It is important to remark that in this study, vascular cell functionality, such as contractility, was not under evaluation, instead functionality of BM-MSCs, known as an excellent cell source for immunomodulation, vascular remodeling and regeneration[54]".

In lines 516-520: "*In vivo* immunogenicity experiments (Fig. S10) demonstrate that the encapsulation of BM-MSCs lowers the inflammation response potentially cause by the implantation. The immunosuppressive cell function is of importance in the design of a new vascular graft, considering that a cause of graft failure is associated to chronic inflammatory response[5]"

Reviewer #3 comment 7d. This reviewer would recommend that the cutaneous implants be struck from the paper, since they do not add value in terms of understanding of the construct, and provide some confusing information.

This section was moved to the supporting information and referred in the main manuscript as an experimental proof of the functionality of encapsulated BM-MSCs and for the possible role in controlling inflammation after implantation. Please see responds to comments 7c.

Reviewer #3 comment 8. For the rabbit carotid implants, it is worthwhile to point out that endotoxin itself can induce endothelial inflammation and hence thrombosis. This may have been why all of the implanted grafts clotted within a short time period. The amount of endotoxin in the constructs should be quantified to gain a better understanding of what is going on here.

The following discussion was included in the discussion section in relation to the possible cause of thrombus formation:

In lines 545-573: "Although patency was only observed after a 12 h period (Fig 8), the new manufactured SDVG has proven suitable in terms of maximal burst pressure (Fig S6), suture retention (Fig S7), hemocompatibility and blood leak-proof grafting demonstrated by in vivo rabbit models (see Fig 8). The rabbit model was applied here as it has been considered adequate for studies of small diameter vascular conduits due to the good similarity in thromboplastic and fibrinolytic properties with humans[78]. Although the diameter of the coronary-like SDVG was adjusted in this study to test the grafting capability in a carotid rabbit model, graft wall thickness and mechanical properties were tailored towards the human coronary artery. Suturing conduits of equivalent diameters but with dissimilar wall thickness, could result in luminal unalignment between the graft and anastomosed natural vessel (see Fig. 8d). This is especially true for 1.5 mm conduits in diameter,

even for a highly skilled and trained vascular surgeon. Unaligned luminal edges generate protruding obstacles for the laminal blood flow vectors at the anastomosis, creating recirculation and stagnation zones. According to previous studies, recirculation and stagnation points intensify the thrombus formation[79]. Although still conjectural, this could be the reason of short patency of SDVG in the rabbit carotid model, considering that even for ePTFE vascular protheses, patency in this model is retained for longer than 1 week[80]. Another possibility could be associated to exacerbated inflammatory reaction and acute thrombogenesis[81] after implantation of the SDVG and triggered by the presence of traces of endotoxin in alginate or GelMA82. Notwithstanding that GelMA was carefully prepared and a low endotoxin alginate selected, SDVG constructs were submitted to endotoxin level quantification and in vivo immunogenicity study utilizing the complete SDVG constructs and its individual components (GelMA, alginate, PCL) (see Fig. S12). Although the endotoxin level present in the SDVG (3.11 EU/ml), which is in the range that a previous study reported induction of inflammatory reaction in macrophages[82], the *in vivo* immunogenicity results showed a low immune reaction for all individual components of the SDVG and the complete SDVG (Fig S12). Therefore, the conclusion that the short-term patency due to thrombus formation is in response to an exacerbated immunoreaction cannot be discarded. In this regard, use of endotoxin-free biomaterials and additional endotoxin control strategies will be required for further translational potential.

Although long-term grafting evaluation is required to demonstrate the efficacy of the bio-inspired SDVG design, these preliminary results show good potential towards clinical usage. Next steps in the development of this SDVG would certainly demand the use of larger and clinically relevant animal models with experimental follow up longer than a year[68,83,84]. "

In this regard, and according to the previous quoted section, we have included the presence of endotoxin as a possible cause of thrombogenesis, and also measured the level of endotoxin in the SDVG construct. Additionally, we have correlated this level of endotoxin with *in vivo* results in which individual material components and the complete SDVG, including the low endotoxin alginate, were tested for immune reaction in the same subcutaneous mice model, not clearly showing the exacerbated increment of lymphocytes counting in dLN as observed in SDVG fabricated with alginate with higher level of endotoxins.

REVIEWERS' COMMENTS:

Reviewer #3 (Remarks to the Author):

The authors have now addressed all of the comments of this reviewer, and the manuscript has been suitably revised.