Biocompatibility and application of carbon fibres in heart valve tissue engineering

Yuan-Tsan Tseng[1,2], Nabil F Grace[3], Heba Aguib[1,2,4], Padmini Sarathchandra[2], Ann McCormack[1], Ahmed Ebeid[4], Nairouz Shehata[4], Mohamed Nagy[4], Hussam El-Nashar[4], Magdi H Yacoub[1,2,4], Adrian Chester[1,2] and Najma Latif[1,2]

[1]Magdi Yacoub Institute, Heart Science Centre, Harefield, Middx, UB9 6JH

[2]Imperial College London, National Heart & Lung Institute, Imperial College, London W12 0NN

[3]Centre for Innovative Materials Research, Lawrence Technological University, Southfield, MI, USA.

[4]Biomedical Engineering and Innovation Laboratory, Aswan Heart Centre, Aswan, Egypt.

* Correspondence:

Corresponding Author: Dr Najma Latif

n.latif@imperial.ac.uk

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Abstract

The success of tissue engineered heart valves relies on a balance between polymer degradation, appropriate cell repopulation and ECM deposition, in order for the valves to continue their vital function. However, the process of remodelling is highly dynamic and species dependent. Carbon fibres have been well used in the construction industry for their high tensile strength and flexibility, and therefore might be relevant to support tissue engineered hearts valve during this transition in the mechanically demanding environment of the circulation. The aim of this study was to assess the suitability of carbon fibres to be incorporated into tissue engineered heart valves, with respect to optimising their cellular interaction and mechanical flexibility during valve opening and closure. The morphology and surface oxidation of the carbon fibres was characterised by scanning electron microscopy (SEM). Their ability to interact with human adipose derived stem cells (hADSCs) was assessed with respect to cell attachment and phenotypic changes. hADSCs attached and maintained their expression of stem cell markers with negligible differentiation to other lineages. Incorporation of carbon fibres into a stand-alone tissue engineered aortic root, comprised of jet-sprayed poly-caprolactone aligned fibres had no negative effects on the opening and closure characteristics of the valve when simulated in a pulsatile bioreactor. In conclusion, carbon fibres were found to be conducive to hADSC attachment and maintaining their phenotype. Carbon fibres were sufficiently flexible for full motion of valvular opening and closure. This study provides a proof of concept for the incorporation of carbon fibres into tissue engineered heart valves to continue their vital function during scaffold degradation.

2

1 Introduction

Tissue engineered heart valves offers the potential to overcome the limitations of current prosthetics. The success of tissue engineered heart valves relies upon several factors: firstly, the scaffold material being strong and flexible enough to withstand the haemodynamic cycle of loading and unloading at a frequency corresponding to range of heart rates during rest and exercise. Second, the construct needs to be receptive to population by cells either seeded during *in vitro* production of the valve or following implantation. Lastly, the scaffold should be biodegradable to allow replacement of hosts' own extracellular matrix (ECM). However, the process is highly dynamic and species dependent (1). Rapid cellular ingrowth and ECM deposition is often observed in animal models, but these observation typically fail to be observed in humans (1–4). Therefore, the potential risk for scaffold degradation and fatigue to occur prior to sufficient laying down of functional ECM leads to structural failure of the constructs.

Textile support as part of heart valve leaflets has been proposed previously by our group and others (5–9). However, most of the literature has been focused on reinforcement of the leaflet with mono or multi-filament yarns, which will still suffer creep, fatigue and unpredictable degradation over time. To ensure stability of the construct during the remodelling process, we have assessed the suitability of incorporating carbon fibres into tissue engineered heart valves to reinforce the biodegradable scaffold. Carbon fibre is a thin fibre between 5 to 20μm in diameter composed of mostly carbon atoms. It has been used in repair of damaged tendons and ligands to provide additional support and strength during surgical repair and regeneration

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64 (10–12). More recently carbon fibres have been used to provide additional strength in

65 scaffold materials used for bone, cartilage and trachea tissue engineered constructs (13–17).

66 The low density and high strength properties of carbon fibres, which are also flexible and

67 have complete elastic recovery after unloading, gives them excellent fatigue resistance (18).

68 This profile of mechanical properties makes them good candidates for inclusion in scaffolds

69 for heart valve tissue engineering.

70

71 Carbon fibres are usually combined with other polymers to reinforce the strength to weight

72 ratio of the composite. This often required surface enhancement on chemically inert carbon

73 fibre surface improves its chemical bonding and adhesion between the carbon fibres and

74 matrix. Plasma oxidisation is a simple and residue free surface activation technique for

75 carbon fibres, thus the focus of this in vitro study was to investigate the biocompatibility of

76 carbon fibres in its pristine and plasma oxidised forms with human adipose-derived stem cells

77 (hADSCs) to determine how binding of cells to the fibres can be maximised. Secondly, we

78 have assessed the flexibility of carbon fibres with respect to motion of tissue engineered

79 valve cusps in a pulse duplicator. We envisage these findings will provide a rationale for

80 further studies into the use of carbon fibres as part of composite scaffold to provide strength

81 and durability of the engineered tissue.

82

83 **2 Materials and Methods**

84 **Carbon Fibres**

4

85   The carbon fibres used in this study were produced by the treatment of a polyacrylonitrile

86   precursor, with pyrolysis, surface treatment and sizing processes (Toray Carbon Fibres

87   Europe, Paris, France). Carbon fibres' size and shape were analysed with a scanning electron

88   microscope (SEM) to assess uniformity of size and shape. For experiments with cells, the

89   carbon fibres were sterilised by incubating in 70% ethanol for 1 hour followed by washing in

90   sterile PBS three times prior to cell seeding.

91

92   **Plasma Oxidation**

93   Carbon fibres were mounted on 24-well CellCrown™24 (Scaffdex Oy) and treated with

94   plasma oxidation at 0.16mbar oxygen (Diener Electronics). Carbon fibres were exposed to

95   30Wfor 10, 20 and 30 minutes, which was compared to 30 minutes of 90W. All following

96   analysis and cell seeding were performed within 24 hours of plasma oxidisation treatment.

97

98   **Cell Culture**

99   hADSCs were purchased from Lonza (PT-5006; Lonza) and cultured in culture medium

100   comprising adipose-derived stem cell basal medium, 10% fetal calf serum (FCS), 1% l-

101   glutamine, and 0.1% gentamicin–amphotericin B (ADSC Growth Medium BulletKitTM, PT-

102   4505; Lonza). The cells were fed every 3 days and sub-cultured at 90% confluency.

103   **Cell Seeding**

104   hADSCs (3 x 10^5 cells) were simultaneously cultured on pristine and plasma oxidised (30W)

105   carbon fibres (fixed on CellCrown™24) for 3 weeks under rotatory seeding at 10 RPM with a

106   rotator (Bibby-Scientific) as described previously (19). In addition, the hADSCs (5000 cells)

107   were seeded on coverslips and cultured for 3 weeks as control. At the end of this period, the

108   coverslips and carbon fibres were washed twice in PBS and fixed in 4% paraformaldehyde

109   for 10 minutes. The fixative solution was removed with three rinses of PBS. The carbon

110   fibres were removed from the CellCrown™24. Cells on coverslips and carbon fibres were

111   permeabilised with Triton X-100 (0.5% v/v in PBS) for 3 minutes and washed two times in

112   PBS-Tween (PBS-T, 0.1% v/v). Cells were blocked using 3% (w/v) bovine serum albumin

113   (BSA) and incubated with primary antibodies (α-SMA (Dako), vimentin (Dako), calponin

114   (Dako); SM22 (Abcam), vinculin (Sigma), EDA-fibronectin (Dinova), alkaline phosphatase

115   (Sigma). CD44 (BD Pharmingen™), Osteocalcin (Abcam), CD105 (Abcam), CD90

116   (Dinova), CD31 (Dako), Sox9 (R&D systems) and PPARγ (Abcam)) in BSA 1.5% w/v for

117   one hour. After thorough washing in PBS-T, the cells were incubated with secondary

118   antibodies for one hour, washed 3 times during 5 minutes in PBS-T and incubated 10 minutes

119   with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Cells were washed again twice in PBS-T

120   and mounted on glass slides in permafluor aqueous mounting fluid (Beckman Coulter,

121   Fullerton, CA). Observations were performed with an inverted confocal microscope (Zeiss,

122   LSM 510 Meta inverted).

123   **Scanning Electron Microscopy**

124 hADSCs grown on coverslips and on carbon fibres were fixed in 2.5% glutaraldehyde in

125 0.1M sodium cacodylate buffer for at least two hours followed by two buffer washes.

126 Specimens were then post fixed with 1% osmium tetroxide in 0.1M cacodylate buffer for 1

127 hour. After two buffer washes, specimens were dehydrated through ascending series of

128 ethanol starting from 25%-100%. Then the specimens were chemically dried using

129 hexamethylene dizilasine (HMDS), mounted on SEM stubs and coated with gold/palladium.

130 Images of carbon fibres with and without cells were taken on JEOL JSM 6010LA analytical

131 scanning microscope. hADSCs on coverslips and on carbon fibres were added to aluminium

132 sample holders with carbon tape, air dried overnight, and coated with Gold/Palladium.

133 Energy dispersive x-ray analyser (EDS) (JEOL JED-2300 X-ray Microanalysis system) was

134 used to investigate the surface structure of the carbon fibres.

135 **Proliferation Assay**

136 After 3 weeks of cell seeding with carbon fibers, proliferation assays were carried out with

137 CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega G-5421) by

138 adding 20μL of MTS/PMS solution with 100μL of DMEM on cells. Plates were incubated for

139 one hour at 37°C, 5% CO_2 and the absorbance was read at 490nm.

140 **Mechanical Testing**

141 Samples of carbon fibres between 3-5mm in length (measured using a calliper (Mitutoyo))

142 and cross-sectional area was measured by SEM (JEOL JSM 6010LA) were subjected to

143 uniaxial tensile testing (TA Electroforce TestBench, Minnesota, USA) at a speed of

144 0.1 mm/s. For each condition, 4 repeated samples, cut longitudinally were measured. The

145 resulting stress strain curve was fitted with six order polynomial trend line. The gradient of

146 elastic modulus was taken from the steepest curve.

Analysis of Cusp Movement/Hinge Mechanism

148 It is well known that carbon fibres suffers brittle snap when bend in 90-degree angles against

149 the direction of the fibres, therefore a motion analysis of a human heart valve were

150 conducted. Aswan Heart Science Centre ethics committee approval and informed consent

151 was obtained to use the CT images from a normal adult individual (female, aged 54 years).

152 The hinge range of movement of the aortic valve was measured using computed tomography

153 (CT) images (Siemens Somatom definition flash dual source multi-slice CT machine with

154 retrospective ECG gating, slice thickness 0.6 mm, pitch 0.18, gantry rotation time 0.28 sec).

155 3D segmentation was used to reconstruct cusp and sinus shape (Mimics Innovation Suite 21

156 research edition, Materialise NV, Leuven, Belgium). The segmented model was rotated to

157 visualise the leaflet and sinus side perpendicular leaflet and sinus plane (side view).

158 3 nadir points of the three sinuses were determined and a plane representing the annular plane

159 was created (Figure 5C). A cross section through the middle of the cusp as a vertical plan

160 (perpendicular to the annular plane) was marked to each sinus and the movement of cusp and

161 sinus wall to this vertical plan was tracked at five points of the cardiac cycle (0, 10, 20, 30

162 and 40%) covering the complete systolic phase.

163 To identify the movement of the hinge, the angles and radii of each cusp and sinus were

164 measured. The radii (R) of the best fit circles are recorded and curvature is calculated by the

165 relation $k=1/R$. A plane (Pp) perpendicular to the annular plane (Pa) through each of the

8

166 nadirs was created to measure the angle of the tangent of each cusp at the nadirs to Pp, and

167 thus, track its movement.

168

169   **Bioreactor Testing**

170 To demonstrate their utility and functionality in a tissue engineered valve construct, carbon

171 fibres were sutured using standard needle into the hinge region of jet-sprayed nanofibrous

172 PCL scaffold (20). The carbon fibres weren't incorporated into the jet spraying process

173 because they are not compatible with the spinning process. In addition, the carbon fibres are

174 only required at regions of high stress to alleviate the stress on the nanofibrous scaffold. The

175 nanofibrous scaffolds were first constructed into a 3D functional valve root using a

176 preparatory process (patent pending) followed by suturing the carbon fibres along the hinge

177 to the belly region and half way up towards the coapting edge in a defined spatial manner (5

178 equally spaced markers were used as a guide in the centre of this region. Each strand consists

179 of 50 individual carbon fibres. The carbon fibres were tethered on outside edge of the

180 commissure and a running stitch was stitched following the marked parallel lines (Figure 6).

181 We sutured the carbon fibres in the radial direction to demonstrate the worst-case scenario in

182 carbon fibre movement in the radial direction. Valve roots with and without embedded

183 carbon fibres were subjected to a hydrodynamic pulmonary profile as set in ISO 5840

184 (20mmHg mean pressure, 70 BPM, 5L/min cardiac output and 35% systolic duration) using

185 the APTUS® bioreactor, (Aptus Bioreactors, USA). High speed camera (500 frames per

186 second) (Sony, UK) was used to capture the opening and closure of the valve over cardiac

9

187   cycles. The relative geometric orifice area was calculated using in-house MATLAB code

188   based on percentage of observed opened area over maximum observable viewing area.

189   **Statistics**

190   Data was tested for normality using the Kolmorogov and Shapiro-Wilk test. A two-tailed t-

191   test was used to test the means between the different groups using GraphPad Prism and a *p*

192   value of <0.05 was considered statistically significant.

193

194   **3 Results**

195   **SEM Demonstrates Carbon Fibres of Uniform Diameter and Structure**

196   The topology of the carbon fibres showed a uniform, smooth and solid structure. It consisted

197   of numerous individual carbon fibres (Figure 1A) with a uniform diameter of 7µm. The

198   carbon fibres had no visible defects such as cracks, pits or splits and no pores (Figure 1C).

199   The cross-section of the carbon fibres showed a solid structure with no internal pores,

200   although some staggering was observed due to uneven cutting and fracturing (Figure 1B).

201   **Plasma Oxidation Disrupts Smooth Surface of Carbon Fibres**

202   Plasma oxidation modified the carbon fibres with an oxide surface layer. The EDX showed

203   oxygen mass increased from 1.4% to 2% with increased treatment time from untreated to 30

204   mins at 30W (Figure 1H). This treatment maintained the smooth surface of the carbon fibres

205   without any signs of damage (Figure 1C to F). However, an enhanced wattage to 90W

206   showed the surface becoming rough with random indentations (Figure 1G) and a marginal

10

207 enhancement of oxide formation to 2.1%. Therefore, it is concluded that 30 minutes of

208 30W plasma oxidation could be administered without any damage to the surface of the fibres.

209 This level of plasma oxidation was used in subsequent cellular experiments with carbon

210 fibres.

211

212 **Morphology of hADSCs on Coverslips and Carbon Fibres**

213 The hADSCs were able to attach and spread on carbon fibres in an aligned and elongated

214 manner, along the length of both pristine and plasma oxidised carbon fibres (Figure 2A to D).

215 In addition, the hADSCs were able to wrap around a single carbon fibre as well as forming a

216 sheet of hADSCs across multiple fibres. Morphology of the hADSCs on the single carbon

217 fibres was dissimilar to hADSCs cultured on coverslips in such that they were elongated and

218 spindly. The hADSCs grown on coverslips showed the typical flattened, spread out

219 morphology with numerous filopodia extending from the cell surface. On reaching

220 confluency, hADSCs made good contact between adjacent cells with some overlapping of

221 cells (Figure 2F).

222

223 **Cell Colonisation to Carbon Fibres**

224 Cell colonisation of hADSCs on the pristine and plasma oxidised carbon fibres was

225 performed with and without dynamic seeding. Static seeding of 3×10^5 hADSCs to the

226 carbon fibres resulted in poor adhesion, which was not quantifiable (not shown). This is most

227 likely due to the settling of hADSCs on the bottom of the well with little contact time to the

11

228   carbon fibres. The dynamic seeding improved contact time of cells to carbon fibres resulting

229   in quantifiable colonisation. The MTS assay showed the cell colonisation on pristine carbon

230   fibres (mean cell number 32662, SD 1609) which was further significantly improved by

231   plasma oxidation (mean cell number 41558, SD 1982), $p=<0.05$ (Figure 2E). The detected

232   cell numbers in the non-plasma and plasma treated carbon fibres are 30K and 40K, Therefore

233   the attachment efficiency is less than 10% as there would be some proliferation.

234

235   **Phenotype of hADSCs on Carbon Fibres**

236   Immunostaining was used to compare the phenotype of hADSCs grown on coverslips,

237   pristine carbon fibres and plasma oxidised carbon fibres (Figure 3 and 4). CD44 and CD105

238   were highly expressed on hADSCs on all 3 formats. CD90, another marker of mesenchymal

239   stem cells, showed strong expression on both forms of carbon fibres. The intermediate

240   filament protein vimentin showed consistent staining of hADSCs on all formats.

241   Differentiation of hADSCs was assessed by using markers for myofibroblastic,

242   adipogenic, chondrogenic and osteogenic differentiation. hADSCs on coverslips showed

243   weak homogeneous expression of SM22 (<20%) and a very low incidence of α-SMA (<10%)

244   -positive hADSCs showed stress fibre staining. This expression was slightly higher between

245   coverslips and the carbon fibres (Figure 4) indicating a low level of myofibroblastic

246   activation. EDA-fibronectin, an early marker of myofibroblastic differentiation, showed

247   enhanced expression on untreated carbon fibres, but a similar low expression on oxidised

248   carbon fibres. Calponin showed a marked increase in expression on untreated carbon fibres.

12

249   There was no expression of CD31 on hADSCs on any format however Sox9 showed weak

250   expression in hADSCs on carbon fibres. There was no expression of PPARγ, osteopontin or

251   alkaline phosphatase. The expression of vinculin was enhanced on carbon fibres (Figure 4).

252

253   **Mechanical Properties of Carbon Fibres**

254   The mechanical testing of carbon fibres was performed with multi-fibre strands to

255   mimic the application scenario. Stress/strain curves were generated (Supplementary Figure 2)

256   using the carbon fibres in the longitudinal direction with the mechanical properties

257   summarised in Table 1. The stress-strain curve showed an initial toe region, which might

258   have resulted due to the initial straightening of multiple carbon-fibres strands. The modulus

259   of elasticity, ultimate tensile stress and failure strain of carbon fibres was 140Gpa (±4.14),

260   3.52Gpa (±0.11) and 0.039 (±0.0036) respectively.

261

262   **Analysis of the Range of Movement of Cusps and Sinuses**

263   An example of a normal human valvular root stained with alcian blue is shown in Fig 5A.

264   The cusp of the valve is hinged onto the sinus wall as part of its structural support. Changes

265   of the angles of each cusp and sinus over the cardiac cycle were measured in the region as

266   shown in Figure 5B, 6C). It showed that the cusps – non (NCC), right (RCC) and left

267   coronary cusp (LCC), had a greater range of motion of 9-70° compared to a limited range of

268   motion, 30-48°, for the coronary sinuses (Table 2).

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                13

270 Measurements of the sinuses' and cusps' radii during valve opening and closure, and

271 consequently of the curvature, showed a maximum range 0.09-0.50 for the left coronary cusp

272 (LCC) and 0.08-0.15 for the corresponding sinus, LCS, (Table 3). Curvature was similarly

273 greater for the non-coronary cusp (NCC) and right coronary cusp (RCC) compared to their

274 corresponding sinuses.

275

276 **Carbon fibre Reinforced Cusp and Geometric Orifice Area of the valve**

277 To demonstrate the proof of principle that carbon fibres can be embedded into PCL sprayed

278 nanofibres and maintained normal valvular cusp function, functional PCL nanofibrous heart

279 valve roots with and without carbon fibres (Fig 6A), were subjected to hydrodynamic testing

280 in a pulse duplicator. The geometric orifice area at the end of the systolic phase in the model

281 without carbon fibres was 65% and this was very similar to the model with carbon fibres at

282 62%. Both models closed fully in the diastolic phase (Fig 6B and C).

283

284 **4   Discussion**

285 In a load bearing application such as the heart valve, biodegradable materials present a

286 significant challenge in balancing the rate of polymer degradation vs continued mechanical

287 function of the construct (2). Therefore, a strategy that incorporates carbon fibres into the

288 tissue engineered constructs to ensure the continued function is proposed here.

289

14

290   Carbon fibre is a well-established material that is currently used in the construction industry

291   such as suspension bridges for its superior strength, fatigue resistance, durability, flexibility

292   and elastic recovery. Thus, a strategic incorporation of the carbon fibres into biodegradable

293   scaffold can ensure the continued load bearing function of the targeted tissue. In addition,

294   previous *in vitro* and *in vivo* studies on other carbon fibres has yielded controversial results

295   showing that carbon fibres induced the growth of new tissue (11,21) and other studies yielded

296   opposite results (22,23). Bone, ligaments and tendon application have previously been the

297   main focus of biocompatibility studies for carbon fibres. With the increased interest in

298   regenerative medicine and tissue engineering, the interaction of carbon fibres with stem cells

299   is now relevant but has not been tested. In this paper we demonstrate that carbon fibres are

300   compatible with hADSCs, support ECM deposition as evidenced by the expression of EDA-

301   fibronectin, have superior strength and are flexible enough to allow the free movement of the

302   valve cusps when stitched into a tissue engineered valve construct from the sinus wall, across

303   the hinge region and into the belly of the cusp. This study has shown the potential for use of

304   these carbon fibres in heart valve tissue engineering.

305

306   We chose to examine the biocompatibility of the carbon fibres with hADSC, since these cells

307   are good candidates in seeding scaffolds for *in vitro* tissue engineering strategies (24). In

308   addition, the differentiation capacity of hADSC, permits these cells to serve as an indicator

309   for conditions that may favour the expression of adipogenic, chondrogenic and osteogenic

310   cell phenotypes (25). The hADSCs were able to adhere to the smooth surface of pristine

311   carbon fibres as shown with the SEM images and the MTS assay (Figure 2). Furthermore, the

15

312   number of cells adhering could be significantly enhanced by prior activation of the surface by

313   plasma oxidation, a process that leads to the production of acid oxides on the surface of the

314   fibres that enhances surface hydrophilicity thereby enhancing surface activation energy

315   suitable for matrix bonding. (26).

316

317   The hADSCs that were cultured onto the carbon fibres retained their stem cell phenotype

318   with no evidence of differentiation into adipogenic, chondrogenic, osteogenic, or endothelial

319   cell phenotypes, However there was some myofibroblastic differentiation with upregulation

320   of α-SMA, calponin and EDA fibronectin. Plasma oxidised carbon fibre reduced this level of

321   activation. The lack of differentiation to other phenotypes indicates that the cells are

322   essentially inert to the carbon fibres as previously reported (27). With respect to *in vitro* heart

323   valve tissue engineering the use of hADSCs and carbon fibres may prove useful especially as

324   hADSCs were shown to retain their phenotype and specific differentiation can be induced

325   and guided by the application of growth factors, peptides, and compounds. We have

326   previously shown that using an active KTTKS peptide motif enhanced the secretion of

327   extracellular matrix components (28) and using specific motifs can drive the expression of

328   tissue-specific extracellular matrix proteins. Combining surface activation with plasma

329   oxidation, carbon fibres can be easily linked to specific bio-active peptides or biomolecules

330   through carbodiimide chemistry.

331

332   The native heart valves have mechanical stiffness in the range 1 to 2 MPa in the radial and 10

333   to 20MPa in the circumferential directions, with UTS of 0.4MPa in radial and 2.6 MPa in

16

334   circumferential direction (29–31) as shown in table 1, and a typical polymeric porous

335   scaffold used in heart valve tissue engineering have significantly lower mechanical stiffness

336   in the range of the 3 to 6MPa and UTS in the range of 0.4 to 0. 7 MPa (30) due to its porous

337   nature to allow for cell colonisation. Furthermore, tissue engineered scaffolds suffer from

338   further deterioration during long implantation periods due to biodegradation and repetitive

339   stress. The engineering application of carbon fibres has been used extensively as a

340   reinforcement component in composite materials due to its ultra-high strength. Therefore, the

341   carbon fibre could be used to form part of a composite scaffold to reinforce it. Mechanical

342   testing of the carbon fibres showed them to have an extremely high modulus of 140GP

343   (±4.14) and ultimate tensile strength at 3.52Gpa (±0.11) in the direction of the fibres. These

344   fall in the range of other carbon fibres produced from polyacrylonitrile (PAN) and mesophase

345   pitch (MPP) (32).

346

347   Despite the high modulus of the carbon fibres, one important design constraint with carbon

348   fibres was that it became brittle and snapped if they were forced to bend in a sharp 90° angle.

349   This has been reported previously when used in reconstruction for chronic anterior cruciate

350   ligaments, where they found that carbon fibres broke under twisting or angular forces (33).

351   The brittleness of the carbon fibres at a sharp 90° angle could be a design constraint for heart

352   valve tissue engineering. As a first step we calculated the angle between the sinus wall and

353   the valve cusp varied during the opening and closing phases of the valve. CT-based analysis

354   of the movement of the aortic cusps in relation to each corresponding sinus showed a great

355   range of movement of the cusps and a maintained curvature at the hinge area, despite the

356   significant changes in angles in the hinge area. These calculations showed that the angle

357   between the sinus wall and each of the three valve cusps did not exceed a 90° angle.

358   Furthermore, as a proof of principle, embedding carbon fibres across the radial direction of

359   the tissue engineered heart valve showed that the geometric orifice area and leaflet motion of

360   a tissue engineered valve in a bioreactor was unaffected by the incorporation of carbon fibres.

361   In this configuration the carbon fibres utilised the sinus wall as a pillar as in a suspension

362   bridge to transfer the load on the valvular cusp during the diastolic phase, while allowing the

363   heart valve to open without significant obstruction.

364

365   This study establishes the potential and utility of carbon fibres in tissue engineered heart

366   valves. There remains a number of additional studies that are required to assess if carbon

367   fibres will provide any benefit to the durability and function of tissue engineered heart valves.

368   The fibres used in this study were sewn into the cusps in radially orientated line across the

369   width of the cusp, these fibres may not necessarily be the optimal width apart or in the best

370   orientation. Further studies are required to establish the potential long-term benefits of the

371   reinforcement of scaffold material on the durability and functions of tissue engineered heart

372   valves both *in vitro* and *in vivo* studies. The current study has used one cell type to assess the

373   biocompatibility of the carbon fibres. Previous studies have also shown carbon fibres to be

374   compatible with cells, but this may be dependent upon the types of carbon fibre used (34–36).

375   Assessment of the cellularisation of scaffold materials containing carbon fibres *in vivo* will be

376   the ultimate test of the biocompatibility.

377

18

5 Conclusion and future work

In this study we demonstrated that carbon fibres can be populated by hADSCs without stimulating their differentiation. Carbon fibres were sufficiently flexible to be incorporated into an *in vitro* functioning tissue engineered valve without restricting the motion of the cusps. Further work is required to optimise the carbon fibre distribution/pattern and embedding method in order to optimise their potential to enhance the durability and hemodynamic performance of tissue engineered valves.

6 Data Availability Statement

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

7 Conflicts of Interest

No conflicts of interest.

8 Author Contribution

YT and NL: experimental plan, concept, data collection, analysis and writing of the manuscript. NG and HA concept, PS, AM, AW, NS, MN and HN: data collection and analysis; MY, AC involves in concept and manuscript editing and review.

9 Funding

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400   **10 Legends**

401   **Figure 1.**

402   SEM images of uniform diameter and smooth surface of carbon fibres. Images of pristine

403   carbon fibres as shown in A) x1000, B) x2000 and C) x10000 magnification shows pristine

404   carbon fibres. Images of carbon fibres with various oxygen plasma treatment, where D) 10

405   min plasma oxidation at 30W, E) 20 min plasma oxidation at 30 W; F) 30 min plasma

406   oxidation at 30W; G) 30 min plasma oxidation at 90 W. Surface remain smooth up to 30

407   mins of the oxygen plasma treatment at 30W, but significant itching were observed with 90W

408   treatment. H) shows the EDX analysis of the carbon fibres surface with increase in oxygen

409   content with increasing the time of plasma oxidation and the wattage

410   **Figure 2.**

411   SEM images of cultured hADSCs on pristine carbon fibre at magnification 1000X (A) and

412   2000x (B). (C) and (D) shows the cultured hADSCs on plasma oxidised carbon fibre at

413   magnification of 1000X and 2000X, respectively. (E) shows the Proliferation (MTS) assay of

414   hADSCs on Pristine and Plasma oxidised carbon fibres (** significant different with $P<0.05$

415   base on two tailed T-test). (F) shows the SEM image of control hADSCs on coverslip at

416   magnification of 2000X.

417   **Figure 3.**

418   Single or dual immune staining of classic markers of hADSCs on coverslips (control), carbon

419   fibres and plasma oxidised carbon fibres cultured over 3 weeks., where blue is nuclei stained

420   with DAPI. immunostaining. Top row is secondary negative control, row 2 shows positive

421   green staining on CD44 and negative red staining of osteopontin, row 3 stains positive for

422   CD105 marker (green) and negative staining for ALP (red) , row 4 stain for positive for

423   CD90 marker (green) and row 5 stain for positive vimentin marker (green).

424   **Figure 4.**

425   Single or dual immune staining of classic markers of hADSCs on coverslips (as control),

426   carbon fibres and plasma oxidised carbon fibres cultured over 3 weeks., where blue is stained

427   with DAPI. immunostaining. Top row shows positive α-SMA (green) and SM22 (red)

428   staining, row 2 shows positive EDA-fibronectin staining (green), row 3 shows positive

429   calponin (green) staining, row 4 shows negative for CD31 (green) and sox 9 (red) staining,

430   row 5 shows positive Vinculin (green) and negative for PPAR-γ (red).

431   **Figure 5.**

432   Cross section through a normal human valvular root stained with alcian blue (blue) and Sirius

433   red (pink) showing the expression of glycoaminoglycans (blue) and collagens (pink)

434   respectively (A). The cusp is on the left and the sinus is on the right. Overlaid CT images

435   through a cross section of a normal human valve at different phases of the systolic cycle (

436   between 0 and 40% of cardiac cycle is shown) (B) and angles which were measured for all 3

437   cusps and sinuses (non-coronary shown in C).

438   **Figure 6.**

21

439   The ventricular view of a tissue engineered valve root without carbon fibre (left) and a

440   prototype of a tissue engineered valve root incorporated with carbon fibre (right) along the

441   hinge area (A). Sample images of the opening and closure of a control valve root (B) and

442   carbon fibre embedded valve root (C) through a cardiac cycle in a puls duplicator. The

443   corresponding graph shows the tracking of their geometric orifice area (GOA) through a

444   cardiac cycle. Both types of valves showing similar maximum GOA at around 60%.

445

446   **11 Tables**

447   Table 1. Mechanical properties of Carbon fibres measured and Human heart valve from

448   literature

Mechanical parameter	Carbon fibre	Heart valve (31)
Modulus of Elasticity	140GPa (±4.14)	0.015Gpa (circumferential) and 0.002GPa (radial)
Failure Strain	0.039 (±0.0036)	0.22 (circumferential) and 0.3 (radial)
Ultimate Tensile Stress	3.52GPa (±0.11)	0.0026GPa (circumferential) and 0.0004GPa (radial)

449

450   Table 2. Angle of the cusps and sinuses formed to the perpendicular line going through the

451   nadir of the annulus at different phases of the cardiac cycles. Keys: NCC: non-coronary cusp;

452   NCS: non-coronary sinus; RCC: right coronary cusp; RCS: right coronary sinus; LCC: left

453   coronary cusp and LCS: left coronary sinus.

22

454

Phase	NCC	NCS	RCC	RCS	LCC	LCS
0%	70°	33°	62°	41°	68°	36°
10%	37°	38°	14°	30°	29°	34°
20%	44°	42°	9°	32°	20°	42°
30%	27°	48°	11°	41°	23°	44°
40%	70°	33°	62°	41°	68°	36°
Range	27-70°	33-48°	9-62°	30-41°	20-68°	34-44°

455

456

457 Table 3. Radius (mm) of the first third of cusp mid-curve and sinus mid-curve. Keys: NCC:

458 non-coronary cusp; NCS: non-coronary sinus; RCC: right coronary cusp; RCS: right

459 coronary sinus; LCC: left coronary cusp and LCS: left coronary sinus.

460

Phase	NCC	NCS	RCC	RCS	LCC	LCS
0%	12	5	11	12	11	6.5
10%	3	6	3	9	2	12
20%	4.5	5.5	4	10	5	8
30%	3	7	2.5	11	2	11
40%	12	5	11	12	11	6.5
Range	3-12	5-7	2.5-11	9-12	2-11	6.5-12

23

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