

1 Characterisation of factors contributing to the performance of
2 nonwoven fibrous matrices as substrates for adenovirus vectored
3 vaccine stabilisation

4 Authors

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34 AVSH and ADD are named inventors on patents relating to adenovirus-vectored vaccines.

35

36 Abstract

37 The global network of fridges and freezers known as the “cold chain” can account for a significant
38 proportion of the total cost of vaccination and is susceptible to failure. Cost-efficient techniques to
39 enhance stability of vaccines could prevent such losses and improve vaccination coverage,
40 particularly in low income countries. We have previously reported a novel, potentially less expensive
41 thermostabilisation approach using a combination of simple sugars and glass micro-fibrous matrix,
42 achieving an excellent recovery of vaccines after storage at supraphysiological temperatures. This
43 matrix is, however, prone to fragmentation and currently not suitable for clinical translation.

44 Here, we report an investigation of alternative, potentially GMP compatible, fibrous matrices. A
45 number of commercially-available matrices permitted good protein recovery, quality of sugar glass
46 and moisture content of the dried product but did not achieve the thermostabilisation performance
47 of the original glass fibre matrix. We therefore further investigated physical and chemical
48 characteristics of the glass fibre matrix and its components. Our investigation shows that the
49 polyvinyl alcohol present in the glass fibre matrix assists vaccine stability. This finding enabled us to
50 develop a custom-produced matrix with encouraging performance, as an initial step towards a
51 biocompatible matrix for clinical translation. We discuss the path to transfer of the technology into
52 clinical use, including potential obstacles.

53

54 Introduction

55 Vaccine immunogens are composed of complex biological macromolecules. Good immunogenicity
56 requires stability of these molecules throughout the lifespan of a product, from production and
57 formulation, through transportation and storage to delivery to the recipient. Extrinsic factors such
58 as light, pH, agitation and oxidants combine with temperature fluctuations to challenge product
59 stability. Therefore, most vaccines need to be continuously stored in fridges or freezers. Maintaining
60 the cold chain and associated logistics in vaccination campaigns can contribute up to 45% of the
61 total cost of vaccination ¹. On the other hand, damage as a result of cold chain breakages costs
62 several million dollars annually ². These issues affect not only low income countries, where the cold
63 chain is regarded as being least reliable, but also developed countries ^{3 4 5}. The vaccines responsible
64 for eradication of smallpox and rinderpest (the only two diseases eradicated by immunisation) were
65 both thermostable, a factor believed to contribute to their success ⁶. Development of technologies
66 to enhance vaccine thermostability has therefore been a major focus of research effort ⁷.

67 Viral vectored vaccines, in particular adenoviruses, are versatile platforms for development of novel
68 vaccines against malaria ^{8 9}, HIV -AIDS ^{10 11}, tuberculosis ¹² and influenza ^{13 14}. They have provided one
69 of the leading approaches to control of the recent Ebola epidemics, and the speed of producing new
70 vaccines using a generic manufacturing process makes them attractive for emergency response to
71 other outbreak pathogens ^{15,16}. Successful thermostabilisation of adenovirus vectored vaccines could
72 thus be valuable for products targeting a wide range of diseases.

73 There have been a number of efforts to enhance stability of adenovirus-based vaccines in liquid
74 formulation ¹⁷⁻¹⁹. Other groups have reported improvement in stability by drying vaccines using
75 lyophilisation ^{20,21}, spray drying ²²⁻²⁴, and nano-patch technology ²⁵. We have previously reported that
76 drying a formulation of adenovirus in glass forming sugars upon a fibrous matrix such as paper or a
77 wetlaid nonwoven fabric permitted full recovery of viable and immunogenic virus, even after six
78 months at 45°C ²⁶⁻²⁸. We refer to this method as sugar-matrix thermostabilisation (SMT). As well as

79 thermostabilisation performance, advantages of SMT include a relatively short process duration (in
80 some cases as little as 18 hours) and avoidance of the stress of extreme in-process temperature
81 exposure involved in lyophilisation and spray drying.

82 Our previous published SMT work used a glass fibre matrix and a polypropylene matrix; we reported
83 that the glass fibre matrix achieved better stability than the polypropylene matrix²⁶. Glass fibre
84 matrix is however prone to shedding of non-biocompatible fibres, and so not suitable for clinical
85 translation. Here, we present results from an effort to characterise the factors contributing to the
86 performance of glass fibre matrix and to test alternative matrices more suitable for clinical use.

87

88 Methods

89 Viruses and infectivity titration

90 Simian adeno virus vectors ChAd63-METRAP and ChAdOx2-RabGP were prepared, purified and
91 tested for quality by the Jenner Institute Viral Vector Core Facility, as previously described^{28,29}.
92 Viruses were dialysed against either a previously used storage buffer (10mM Tris, 7.5 % w/v sucrose,
93 pH 7.8) or unbuffered 0.5M trehalose and sucrose and stored at -80°C as stock. Typical preparations
94 were supplied and stored at a titre of c. 1×10^{12} virus particles (VP) per mL, corresponding to c. 1×10^{10}
95 infectious units (IU) per mL and hence a particle: infectivity ratio of c. 100.

96 For infectivity titration, duplicate fivefold serial dilutions were prepared in complete DMEM (10%
97 FCS, 100 U penicillin, 0.1 mg streptomycin/ml, 4 mM L-glutamine) and used to infect 80-100 %
98 confluent HEK293-TREx cells (ThermoFisher) grown in 96-well plates (BD Purecoat Amine, BD
99 Biosciences, Europe). Infected cells were immunostained and imaged as previously described³⁰.
100 Wells containing 20-200 spots were used to back-calculate recovered infectious units.

101

102 Drying, thermochallenge and reconstitution

103 Stock vaccines were thawed and diluted into unbuffered 0.5M trehalose – sucrose solution. Dilution
104 factors, final viral titres, and the trehalose: sucrose ratio varied between experiments, as stated in
105 figure legends.

106 Fibrous matrix was cut into 100 mm² pieces, loaded with vaccine formulation and transferred to a
107 glove box (Coy Laboratory Products). An activated silica bed within the chamber and circulation of
108 air through desiccation capsules containing anhydrous calcium sulphate (Drierite™, W.A. Hammond
109 Drierite Co.), regulated by a humidity controller (Series 5000, Electro-tech Systems), was used to
110 maintain relative humidity beneath 5%. A portable datalogger (AET-175, ATP instruments) was used
111 for recording changes in relative humidity and temperature during the desiccation process. The
112 temperature inside the enclosed glove box remained between 22°C and 25°C for all experiments.

113 Samples were transferred into 2 mL glass vials, stoppered and crimp sealed under dry conditions
114 within the glove box prior to further use. Samples undergoing thermochallenge i.e. storage at
115 elevated temperature (typically 45°C for one or four weeks) were stored within secondary packaging
116 (moisture barrier bags).

117 For experiments involving reconstitution of dried samples, this was performed by addition of
118 phosphate buffered saline (Sigma), followed by brief vortexing of the vial (1±0.5 seconds, three
119 times). Virus infectivity after reconstitution was assayed as described above. Recovery of infectious
120 virus was quantified by comparison to a sample of the starting material, included on the same assay
121 plate. Between the set-up of an experiment and the assay of recovered infectivity, such comparator
122 material was stored at -80°C in aqueous buffer (under which conditions loss of infectivity is known to
123 be negligible).

124 Recovery was calculated in terms of log₁₀-fold loss in the total infectious virus content of the matrix
125 i.e. log₁₀-fold loss = log₁₀(infectious units dried on matrix based on -80 stored sample) –
126 log₁₀(infectious units recovered from matrix). Unlike viral titres (infectious units / mL), this
127 parameter is independent of the volumes in which sample was dried or reconstituted. 0.3 log₁₀-fold
128 loss thus implies c. 50% recovery, 0.5 log₁₀-fold loss implies c. 30% recovery, and 1 log₁₀-fold loss
129 implies 10% recovery.

130

131 **Karl Fischer moisture analysis**

132 Residual moisture content in single matrix post-desiccation was determined with a Karl Fischer
133 moisture analyser equipped with coulometer (Metrohm), and Hydranal-Coulomat titration solution
134 (Honeywell, Fluka) in accordance with the manufacturers' recommendations. A standard was used
135 to calibrate the instrument performance (lactose standard 5%, MerckMillipore). Residual moisture
136 content was expressed as the mass extracted per 50 µL of formulation loaded into each matrix.

137 Measurement of subvisible particles

138 100 mm² pieces of glass fibre (S14) were cut, autoclaved at (121 °C, 15 minutes) and loaded with 50
139 µL of sugar solution prior to drying in the glove box, vialling and reconstitution as described above.

140 Reconstituted solution from 10 vials was aspirated using a syringe with a conventional 20G needle
141 (BD Biosciences), to produce a single pooled unfiltered sample. Reconstituted solution from a
142 further 10 vials was aspirated using a 5-micron filter needle (BD Biosciences), to produce a single
143 pooled filtered sample. Both pools were diluted to a final volume of 25 mL and tested for sub-visible
144 particles by light microscopy according to Ph.Eur (2.9.19) / USP <788> by a commercial testing
145 laboratory (Reading Scientific Services Limited).

146

147 Scanning electron microscopy

148 Untreated matrices (i.e. without sputter coating) were loaded onto aluminium mounts using carbon
149 conductive tabs and imaged using a Zeiss-Evo LS15 variable pressure scanning electron microscope
150 (SEM) equipped with variable pressure secondary electron detector (Carl Zeiss Ltd). Imaging was
151 performed at a chamber pressure of 50 Pa air and accelerating voltage of 15 kV. Further analyses of
152 images such as measurement of fibre diameter were made using Image J software. At least 40
153 measurements per matrix were performed, using images taken at varying magnification.

154

155 Differential scanning calorimetry

156 The glass transition temperature (T_g) of sugar glass in matrices was measured immediately after
157 drying had completed using Differential Scanning Calorimetry (DSC). The melting point of the binder
158 in untreated glass fibre (S14) was measured using DSC (Q2000, TA instruments). The instrument was
159 purged with dry nitrogen (50 mg/mL) continuously during sample measurement. Calibration was
160 performed prior to measurements using a certified reference material (Indium) for temperature and
161 heat flow accuracy.

162 Multiple 6-millimetre diameter discs were cut out of a matrix dried with 0.5M trehalose: sucrose
163 (50:50). Discs were weighed and, for each matrix type in turn, a total mass of 5-15 milligrams was
164 loaded into Aluminium DSC pans (TA Instruments) and hermetically sealed. Samples were subjected
165 to a temperature ramp from -20 °C to 180 °C at a heating rate of 10 °C per minute. Measurements
166 on all samples were performed in duplicates. Thermograms relating heat flow (W/g) to temperature
167 (°C) were analysed using Trios software (TA Instruments) for identification of the glass transition (T_g)
168 onset temperature.

169 For the measurement of enthalpic recovery, which manifests as an endothermic peak at the glass
170 transition, modulated DSC (temperature modulation $\pm 0.50^{\circ}\text{C}$ every 60 seconds and ramp rate
171 $3^{\circ}\text{C}/\text{min}$ from -20°C to 100°C) was employed. The enthalpic recovery was estimated by linear peak
172 integration in the thermograms plotted between nonreverse heat flow (W/g) and temperature (°C)
173 using Universal Analysis software (TA Instruments).

174

175 Protein recovery

176 A 10mg/mL solution of lysozyme (Sigma-Aldrich) was made in 0.5M trehalose sucrose. 25 μL was
177 loaded into each matrix in triplicates. Protein was reconstituted from the matrices after desiccation
178 overnight and recovery was quantified using EnzChek Lysozyme Assay Kit (ThermoFisher Scientific).
179 Fluorescence measurements were performed in triplicates for each sample and protein recovery
180 calculated by interpolation on a standard curve, using GraphPad Prism.

181

182 Thermogravimetric analysis

183 Degradation temperatures of matrix constituents were measured by thermogravimetric analysis
184 using a TGA Q500 (TA instruments). Samples loaded into a tared platinum pan just prior to
185 measurement were subjected to a temperature ramp at 5°C per minute from ambient to 550°C in a
186 flowing nitrogen atmosphere (100ml/min). The gas was switched to air at 550°C (100 ml/min) and

187 heat was continued at the rate of 5°C/min to 730°C. Data was analysed using Universal Analysis
188 software (TA Instruments).

189

190 Polyvinyl alcohol (PVA) extraction and Fourier transform infrared spectroscopy

191 A 500 mm X 27 mm piece of glass fibre (S14) was dissolved in 100 mL of ultrapure water by stirring
192 and heating at 90 °C for 48 hours. The suspension was filtered through a 0.2 µm filter and the filtrate
193 was freeze dried (Virtis AdVantage 2.0, SP Scientific) to a white amorphous material. This residue
194 was subjected to single reflection attenuated total reflection (MIRAacle™, Pike Technologies)
195 Fourier transform infrared spectroscopy ATR-FTIR (Tensor 37, Bruker) equipped with nitrogen-
196 cooled mercury cadmium telluride detector.

197 To obtain a spectrum of the sample, an average of 64 interferograms was collected at a resolution of
198 4 cm⁻¹ in the wavelength range from 4000 cm⁻¹ to 750 cm⁻¹ and blank subtracted.

199 Spectra were analysed using OPUS 6.5 software (Bruker) and compared with RMIT University's
200 spectral library of organic compounds generated using Spectrum 10 software (Perkin Elmer).

201 Fingerprint spectra shown in Figure 4D were prepared using GraphPad Prism.

202

203 Dynamic light scattering (DLS)

204 The molecular weight of the PVA binder present on the glass fibre matrix (S14) was estimated by
205 DLS. PVA was extracted and freeze-dried as described above. Solutions of this extracted sample and
206 standards of known polymer size were prepared in water to the concentration of approximately 1.3
207 mg/mL and passed through 0.22 µm and 300 kDa molecular weight cut-off filters. The sample was
208 then concentrated approximately five-fold from 4mL to 0.75 mL using a 3KDa MWCO filter (Vivaspin,
209 GE). Zetasizer Nano ZS and DTS software (Malvern Instruments) was used for measurement of
210 hydrodynamic diameter based on size distribution by volume. Independent duplicate preparations
211 of all standards and samples were tested. GraphPad Prism was used to generate a standard curve

212 plotted between measured hydrodynamic diameter and known average molecular weight (KDa) to
213 interpolate size of the PVA in the extract.

214 Nuclear magnetic resonance (NMR) spectroscopy

215 The degree of hydrolysis of the PVA binder present on the glass fibre matrix (S14) was estimated by
216 ¹H NMR measurement as the intensity of the peak attributable to the acetyl group present in non-
217 hydrolysed PVA. A 5 mg/mL aqueous solution of PVA extracted from the glass fibre sample (S14) was
218 prepared in deuterium oxide. Reference spectra for PVA with varying degrees of hydrolysis were
219 obtained by mixing >99% hydrolysed PVA and 80% hydrolysed PVA in appropriate proportions to
220 produce standards containing c. 0%, 2%, 5%, 10% and 20% acetyl groups, again at 5 mg/mL in
221 deuterium oxide. An AVIII 700 instrument (Bruker Biospin) was used to generate ¹H 1D spectra
222 (employing a quantitative 1D NOESY (Nuclear Overhauser Effect Spectroscopy) presaturation
223 sequence with recovery delay $d_1 = 30$ s) and 2D ¹H-¹³C HSQC (heteronuclear single quantum
224 coherence spectroscopy) plots.

225 Application of PVA to matrices

226 Aqueous solutions of each type of PVA to be investigated were prepared at 10 mg/mL. Matrices
227 were cut into approximately 100 mm² pieces and loaded with until the matrices were saturated by
228 the solution and air-dried overnight. The polyamide matrix (33100L) required surface modification
229 by washing in 100% ethanol for 20 minutes and air drying before PVA could be loaded. Following
230 PVA application, vaccines were dried on the matrices and thermostability assessed as described
231 above.

232 Study of physical characteristics of matrices

233 The physical characteristics of matrices were studied using NWSPs (Nonwoven Standard Procedures)
234 prescribed by the EDANA, the international trade association for the nonwoven industry.

235 Areal density was measured as ratio of mass in grams and area in square metres according to EDANA
236 NWSP 130.1R0 (15).

237 Matrix thickness was measured at an applied pressure of 0.5 kPa according to EDANA NWSP
238 120.1.R0 (15) using a thickness tester (ProGage, Thwing-Albert Instrument Company).

239 Fibre length was measured based on 60 individual fibres extracted from the matrix using a Leica MD
240 G41 optical microscope in transmission mode and Image J software.

241 Minimum mean and maximum pore size were obtained using a POROLUX 100 Automated Capillary
242 Flow Porometer using Galpore liquid of surface tension 15.6 mN·m⁻¹. A total of five measurements
243 were taken per sample. Porosity (ϵ) of the GE S-14 was determined by the equation $\epsilon = (1 - \phi) \times 100\%$
244 where ϕ is the volume fraction measured as a ratio of bulk matrix density to bulk fibre density.

245 The surface tension of aqueous sugar solution on the matrix was measured on a Kruss K100
246 tensiometer using the Wilhelmy plate method in which the force exerted on a suspended plate
247 when it touches the surface of a liquid is related to the surface tension and the contact angle
248 according to the equation $\sigma = \frac{F}{L \cdot \cos\theta}$ where σ = surface tension of the liquid, F=measured force,
249 L=wetted length, and θ =contact angle.

250 Wettability was evaluated using the Washburn method, again on a Kruss K100 tensiometer. The rate
251 of mass uptake when the porous substrate (matrix) comes in to contact with a liquid was used to
252 determine the capillary constant of the substrate by applying the Washburn equation $\frac{m^2}{t} = \frac{c \cdot \rho^2 \cos\theta}{\eta}$,
253 where m=mass, t=flow time, c=capillary constant of the nonwoven, ρ =density of the liquid, σ =
254 surface tension of the liquid, θ =contact angle, η =viscosity of the liquid. The capillary constant of the
255 matrix was determined using n-hexane which has a contact angle of 0°.

256 Development of Nonwoven Fabrics for Vaccine Thermostabilisation

257 A glass fibre matrix (Figure 5B) was custom made by a wet-lay process using commercially available
258 glass fibre and polyvinyl alcohol aiming for similar porosity, thickness, areal density and wetting
259 behaviour as the glass fibre sample (S14). A matrix of glass fibre type 475 with diameter 4 μm (Johns
260 Manville) was prepared and 1 g/L PVA solution (>99% hydrolysed, Mw 146000-186000 kDa, Sigma)
261 was applied to each side using a spray gun before drying at 110°C for 15 minutes.

262

263 Results

264 Glass fibre matrix achieves good stability but is not suitable for clinical

265 development

266 In our previously published work, we had used low vaccine doses (1.1×10^{10} viral particles per
267 matrix, as compared to a typical human dose of 5×10^{10} viral particles), applied to the glass fibre
268 matrix 'Standard 14' (S14, GE Healthcare, Figure 1A) ²⁶. We speculate that the fibrous matrix
269 provides a high surface area, favouring relatively rapid drying of the product despite the gentle
270 conditions used (18-24 hours at 20-23 °C and atmospheric pressure). The process results in films of
271 vitreous 'sugar glass' embedded in the matrix (Figure 1B). As previously reported, this 'base-case'
272 process achieves a sugar glass transition temperature (T_g) after drying of 47-55°C, with moisture
273 content 3-5% of total solute dry weight ²⁶.

274 We have now dried full human doses (5×10^{10} virus particles, approximately 5×10^8 infectious units
275 [IU]) of a simian adenovirus vectored rabies vaccine (ChAdOx2 RabG) upon 1 cm² of the matrix, with
276 <0.1 log₁₀-fold loss in-process (i.e. during desiccation) and approximately 0.3 log₁₀-fold loss after
277 thermochallenge at 30°C for a month (Figure 1C) ²⁸. For explanation of the log₁₀-fold loss metric,
278 please see Methods.

279 Despite the good thermostabilisation performance of the glass fibre matrix, concerns were raised
280 regarding the brittleness of glass fibre would result in shedding of fibres during the process of
281 vaccine reconstitution. Indeed, macroscopic damage to the matrix was sometimes apparent at the
282 point of reconstitution. We therefore sought to quantify subvisible particles (0.1-100 micron) in the
283 reconstituted vaccine. Application of a pharmacopoeial light microscopy method revealed numerous
284 glass fibres (Figure 1D). Although passing the reconstituted solution through a 5 µm filter needle
285 removed virtually all detectable glass particles, reducing the particulate burden within
286 pharmacopoeial limits, such a method is unsuitable for clinical development, with the possible
287 exception of very early-phase studies ³¹.

288 Selection of commercially-available matrices for evaluation

289 In our previously published work, we reported that the S14 matrix achieved better vaccine
290 thermostability than an alternative commercially-available polypropylene-based matrix (HDC®II J200,
291 Pall Corporation). We therefore sought to identify alternative commercially-available matrices which
292 might offer thermostability equivalent to or better than that achieved with S14, but without the
293 problem of shedding of non-biocompatible fibres. A set of seven matrices were selected based on
294 manufacturers' product specifications claiming low fibre shedding, low chemical leaching and
295 compatibility with sterilisation either by dry heat, steam or gamma-radiation sterilisation (Table 1).
296 Henceforth matrices are referred to, for clarity of identification, in terms of their fibre material and
297 the manufacturer's product name.

298 We initially screened all matrices for suitable loading capacity ($>20 \mu\text{L}$ of deionised water/cm²).
299 Matrices which did not absorb $20 \mu\text{L}/\text{cm}^2$ (without visible remaining beads of water within two
300 seconds) were re-tested after treatment with 2% polysorbate 20 solution. Matrices which did not
301 absorb $20 \mu\text{L}/\text{cm}^2$ after detergent treatment were not studied further. We proceeded to further
302 study of the remaining five matrices along with the two previously tested matrices (glass fibre S14
303 and polypropylene J200).

304 The architecture of the selected matrices was characterised by scanning electron microscopy (Figure
305 2). Fibre diameters estimated for each matrix type using Image J analysis are presented in table 1.

306 Fibres in the polypropylene (J200) matrix (Fig 2A) and glass fibre (conjugate pad) matrix (Fig 2F)
307 shared the straight, rod-like fibre morphology seen in the original glass fibre (S14) matrix (Fig 1A-B)
308 but had larger fibre diameters of 10 – 20 μm (as compared to 4 μm in the S14 glass fibre sample).
309 The remaining four matrices (Fig 2B-E) exhibited a greater degree of curl along their length. A film,
310 potentially a binder, was apparent on the glass fibre matrix (conjugate pad) (Fig 2F). Matrices loaded
311 with 0.5 M sugar solution and dried at room temperature for 24 hours also showed differences in

312 the distribution of the sugar glass intercalated between the fibres **Error! Reference source not**
313 **found.**(Figure 2). Distinct films of sugar glass between the fibres were visible on the two glass fibre
314 matrices, but not in the polyamide (33100L), polyester (leukosorb) or polypropylene (J200) matrices.
315 The sugar loaded polyester (23100) matrix and cellulose (31 ET CHR) matrices showed a glazing
316 effect on the fibres, with discrete sugar glass films being apparent.
317

318 **Thermostability of adenovirus vaccine formulated in commercially-available** 319 **matrices**

320 Adenovirus vaccine vectors were formulated in 0.5M TS and dried on the five selected 'new'
321 matrices, with glass fibre (S14) and polypropylene (J200) matrices as comparators of known
322 performance. Dried matrices were thermo-challenged for a week at 45°C prior to reconstitution and
323 infectivity titration. Marked thermostabilisation performance differences between the matrices
324 were apparent (Table 1).

325 As previously observed, the glass-fibre matrix (S14) showed minimal (less than 0.3 log₁₀-fold) loss of
326 infectivity compared to the -80 °C stored positive control. None of the alternative matrices matched
327 this level of performance: the next-best-performing matrices were polyamide (33100L) and glass
328 fibre (conjugate pad), with infectivity loss of 0.9 log₁₀-fold in each. The cellulose-based matrix (GE)
329 performed most poorly.

330

331 **Relationships between matrix type, thermostabilisation performance and** 332 **physical characteristics of sugar glass**

333 In order to understand the highly variable thermostabilisation performance of the matrices, we
334 investigated whether performance could be correlated with the physical properties of the matrices
335 or the sugar glass formed.

336 Standard sugar formulation (0.5M trehalose sucrose) was loaded into each matrix and dried at room
337 temperature and < 5% relative humidity. Dried samples were then subjected to modulated
338 differential scanning calorimetry (DSC) to measure glass transition temperature (T_g) onset
339 temperature and enthalpic recovery of the sugar glass formed on the matrices. The glass transition
340 temperature (T_g) indicates the temperature at which a low mobility sugar glass changes to a highly
341 mobile rubbery state and is known to be related to product stability in dry formulations³². Enthalpic
342 recovery is a measure of energy dissipated as a glass progresses through equilibrium and can reflect
343 molecular rearrangement during storage or physical ageing³³.

344 The T_g was similar for all matrices, observed over a narrow range between 52°C and 56°C, and did
345 not correlate with thermostability (Figure 3A). A possible correlation was observed between
346 thermostabilisation performance and high enthalpic recovery (Figure 3B, $r^2=-0.70$, $p=0.02$), as seen
347 with glass fibre matrices conjugate pad and S14 followed by polypropylene (J200) based matrix.
348 There was substantial variation in the residual moisture content of products dried under the same
349 conditions on different matrices (Figure 3C), with lowest residual moisture in the best performing
350 matrix, S14. Recovery of a model protein (lysozyme) after desiccation and reconstitution did not
351 predict thermostabilisation performance (Figure 3D).

352

353 **Characterisation of S14 glass fibre matrix**

354 Given our inability to identify a commercially-available matrix suitable for clinical application, we
355 turned our attention to detailed characterisation of the best-performing S14 matrix, with a view to
356 future production of a similar matrix from biocompatible materials.

357 We initially performed a more extensive characterisation of the physical properties of the matrix,
358 with results as shown in Table 2.

359 Use of chemical binders is common in the production of nonwoven fabrics such as S14 to provide
360 strength and desirable surface properties. In view of the possibility that a binder might be
361 contributing to S14's thermostabilisation performance, we investigated whether such a binder could
362 be identified in the matrix.

363 Scanning electron microscopy demonstrated film-like material which could represent binder
364 covering fibres in some sections of the S14 sample (Figure 4A). Differential scanning calorimetry and
365 thermogravimetric analysis (Figures 4B-C) demonstrated the presence of a material with a melting
366 point of 220°C and a degradation temperature at 260°C.

367 For further characterisation, the binder was extracted in liquid and freeze dried. Fourier transform
368 infrared (FTIR) spectroscopy of the extract provided a fingerprint spectrum, which matched closely
369 with the expected spectrum of polyvinyl alcohol (PVA) (Figure 4D)^{34,35}.

370 The solubility and other properties of PVA vary widely according to molecular weight (MW) and
371 degree of hydrolysis, and so we sought to further characterise the presumed PVA extracted from
372 S14. We used dynamic light scattering (DLS) to compare the hydrodynamic radius of the PVA extract
373 to those of PVA samples of known MW. The results were consistent with a MW in the range of 35
374 kDa (Figure 4E).

375 We then used ¹H NMR to estimate the degree of hydrolysis of the polymer. The ¹H NMR spectrum
376 obtained (Figure 4F) was consistent with PVA, with a 2:1 ratio of the areas under the peaks at ~1.6
377 ppm and 3.9 ppm (corresponding to hydrogens in the CH₂ and CH environments respectively).
378 Results obtained using a range of standards of varying percentage hydrolysis showed a clear
379 relationship of the size of a peak at 2.05 ppm to the acetyl group content (the presence of which, in
380 a sample of PVA, indicates incomplete hydrolysis). The spectra of the completely hydrolysed
381 standard and the S14 extract were similar, with only a trace of a peak in this area, and so we
382 concluded that the PVA extracted from S14 is likely to be completely hydrolysed.

383

384 **PVA enhances adenovirus stabilisation by SMT**

385 Having identified PVA in the best-performing matrix, S14, we investigated whether the PVA may
386 function not only as a binder but might actually contribute to vaccine thermostabilisation.

387 We reasoned that treatment with PVA might enhance the thermostabilisation performance of
388 relatively poorly-thermostabilising matrices. We observed that application of PVA extracted from
389 glass fibre (S14) significantly improved thermostabilisation performance of two of the three tested
390 matrices, as assessed by infectious virus recovery after a four week 45 °C thermochallenge. Recovery
391 from the polyester matrix (Leukosorb) was enhanced by 1.3 log₁₀-fold, while enhancement from the

392 cellulose matrix (ET CHR) was enhanced by 2 log₁₀-fold (Figure 5A). Addition of PVA to a glass fibre
393 matrix (Pall's conjugate pad) was not beneficial. This matrix already contains a binder (Fig 2F),
394 possibly PVA.

395 We proceeded to test whether the degree of hydrolysis of PVA had any impact on vaccine
396 thermostability at 45°C for an extended period of 28 days. We tested polyester (Leukosorb),
397 polyamide (33100L) and a custom-made glass fibre-based matrix after treatment with PVA extracted
398 from S14 and two other commercially sourced polymers (Sigma) which were similar in size but
399 differed in degree of hydrolysis (30-70 KDa / 87-90% hydrolysed, and 31-50 KDa/ >99% hydrolysed).
400 We again observed substantial improvements in thermostabilisation performance, exceeding a 1
401 log₁₀-fold increase in infectious virus recovery after thermochallenge, when PVA was applied to
402 matrices which did not contain PVA at baseline (polyamide and polyester) (Figure 5B). The greatest
403 enhancement was seen with the S14 extract, followed by the fully hydrolysed PVA, with the least
404 enhancement seen with the incompletely hydrolysed PVA (Figure 5B).

405

406 [Towards development of custom nonwoven fabrics for vaccine](#)

407 [thermostabilisation](#)

408 Having characterised the geometry and composition of S14, and the contribution of PVA to its
409 thermostabilisation performance, we proceeded to develop a bespoke matrix 'in-house'. Although
410 our ultimate aim is to produce biocompatible matrices, we sought as an initial step to test whether
411 the understanding we had gained would enable us to produce an 'in-house' wet-laid glass fibre
412 matrix which could replicate S14's thermostabilisation performance. The new matrix had structural
413 properties similar to those of S14 (area density 50.6±1.5 g.m⁻², thickness 0.56±0.02 mm, mean flow
414 pores 25±2.3 µm, absorption capacity 11.1±0.3 g/g, composed of borosilicate glass fibres with
415 diameter 3.6±1.8 and length 1.3±0.6 mm; see table 2 for data relating to S14), and a similar
416 appearance (Figure 5C). Although thermostabilisation performance of the custom-made matrix did

417 not exactly match that of S14 (Figure 5D), it was closer than had previously been achieved with any
418 of the other tested matrices (Fig 5B). As expected, further modification of the matrix with additional
419 PVA had no effect.

420 Discussion

421 The starting point for the present study was the observation, in our previous work, that the non-
422 biocompatible glass-based S14 matrix out-performed a polypropylene matrix ²⁶. This posed the
423 question of which properties of the matrix could be relevant for SMT performance, and whether a
424 more suitable matrix than S14 could be identified for clinical translation.

425 Our initial attempts to find a suitable commercially available matrix yielded disappointing results.
426 Adenovirus stability on the tested matrices was poor (table 1). Multiple variables differed between
427 each matrix, and we were therefore unable to perform experiments to clearly isolate the effect of a
428 single variable. There was not a clear relationship between characteristics of the matrices and their
429 stabilisation performance (Figures 2 and 3), with the possible exception of high enthalpic recovery of
430 sugar glass (which is not a parameter which can readily be 'designed in' to a new matrix).

431 Our ability to use analysis of commercially available matrices to draw conclusions to guide design of
432 new biocompatible matrices was thus limited. We therefore changed our strategy, seeking to
433 characterise S14 in detail and produce similar matrices 'in-house', allowing us to identify features of
434 S14 contributing to its stabilising performance.

435 Most significantly we found that PVA, present on the S14 matrix as a binder, appears to contribute
436 to the stability of adenovirus (Figures 4 and 5). It was shown that fully hydrolysed PVA, similar to
437 that we extracted from S14, was most beneficial in thermostabilisation (Figure 5). Polyvinyl alcohol is
438 potentially suitable for use as an excipient in vaccine formulations: it is 'generally regarded as safe'
439 (GRAS) and is also a FDA approved inactive ingredient for parenteral use ³⁶. PVA has previously been
440 explored as an excipient in a number of studies of bio-macromolecular stability. It has been found to

441 be beneficial in some formulations of proteins, including insulin ³⁷, but benefit has not been seen
442 consistently in other studies ^{38 39 40}. PVA may contribute to protein stability by hydrogen bonding of
443 hydroxyl groups in PVA to the proteins. In dried protein formulations, PVA has also been shown to
444 prevent deamidation more potently than another widely used polymeric excipient, polyvinyl
445 pyrrolidone ⁴¹.

446 We now intend to develop the SMT method towards clinical application. There are two principal
447 obstacles to this goal: robust and GMP compliant execution of the process, and the availability of a
448 suitable matrix for GMP production. Robustness and GMP compliance of the process are clearly
449 interlinked. We believe several of the challenges of GMP execution of the SMT process can be
450 addressed by execution of drying within a lyophilizer without freezing or the application of vacuum:
451 existing large-scale GMP lyophilisation facilities could provide the necessary controlled temperature,
452 low humidity, aseptic environment. With respect to robustness, we have found in recent work that
453 the stability achieved by the process can be highly sensitive to deviations from the intended
454 conditions and, more troublingly, some unexplained inconsistency in performance can occur: work is
455 ongoing to address these issues. With respect to the matrix, GMP execution is likely to require
456 development of a new non-woven. In addition to replicating the stabilising performance of S14, such
457 a matrix needs to be biocompatible, to have good mechanical integrity (in particular, without
458 shedding fibres into the reconstituted product), and to be produced in line with the quality
459 requirements for a GMP raw material. We are now using the data provided by the present study in
460 order to develop such a matrix, mimicking the physical properties of S14 and making use of the
461 beneficial effect of PVA, but without the problematic use of glass.

462 [END]

463

464 Figure Legends

465 Figure 1: SMT on S14 results in thermostable vaccine embedded in sugar glass,

466 Panels A and B show scanning electron micrographs of S14, respectively before and after loading and
467 drying sugar formulation. In Panel A, the arrangement of fibres in S14 is apparent (200X
468 magnification, 100 μm scale bar). In panel B, sugar glass films are visible between fibres (main panel
469 at 200X magnification with 100 μm scale bar, inset at 1000X magnification with 20 μm scale bar).

470 Panel C shows recovery of vaccine from matrix loaded with a human dose of adenovirus, both
471 immediately post-drying and after thermochallenge at 30 °C for one month. Results plotted illustrate
472 total virus recovery from each replicate matrix (n=4 for -80 °C comparator, n=2 for post-drying, n=3
473 for post-thermochallenge), with lines indicating the mean of the replicates. Numbers above each
474 result show \log_{10} -fold loss from the -80 °C control.

475 Panel D shows a representative light microscopy image of fibres shed from S14 after vaccine
476 reconstitution (scale bar 200 μm).

477

478 **Figure 2: Physical appearance of fibres and sugar glass formed in matrices**

479 Scanning electron microscopy images of six different matrices at 1000X magnification. Within each
480 panel, the left-hand image shows empty matrices and the right-hand image shows sugar-loaded
481 fibres. Scale bar shows 20 μm in each image.

482

483 **Figure 3: Physical properties of sugar glass have differential impact on vaccine**
484 **thermostability**

485 Each panel relates the thermostabilisation performance of the various matrices (after one-week
486 thermochallenge at 45 °C, Y-axis, data shown in Table 1) to a potentially explanatory variable on the
487 X-axis: glass transition temperature (Panel A); enthalpic recovery (Panel B); moisture content (Panel
488 C); and protein recovery (Panel D). Results of Pearson correlation analysis are shown within each
489 panel. DSC measurements (Panels A and B) were made in singlicate, Karl-Fischer measurements
490 (Panel C) were made in duplicate, and protein recovery measurements (Panel D) were made in
491 triplicate. For panels C and D, points represent the mean measurement.

492

493 Figure 4: Chemical properties of glass fibre matrix

494 Panel A shows a scanning electron microscopy image indicating presence of binder in glass fibre
495 (S14), visible as a film in the white-circled area. Scale bar shows 10 μm in each image. Such areas
496 were relatively sparse, compared to the extensive films apparent in sugar-loaded matrices (Figure
497 1B).

498 Panel B shows a differential scanning calorimetry thermogram of glass fibre (S14), representative of
499 duplicate measurements. The graphs show heat flow as a function of temperature during scanning
500 from 40 $^{\circ}\text{C}$ to 250 $^{\circ}\text{C}$.

501 Panel C displays thermogravimetric analysis of glass fibre (S14), showing a thermal decomposition
502 step. Weight losses (green lines) and the rate of weight loss (i.e. derivative, $\%/^{\circ}\text{C}$) (blue lines) are
503 shown.

504 Panel D shows the sample spectrum obtained from attenuated total reflectance Fourier transform
505 spectroscopy (ATR-FTIR) for the binder recovered from glass fibre (S14) (black), and a reference
506 library spectrum for PVA (grey).

507 Panel E shows estimation of the molecular weight of the binder extracted from glass fibre (S14) by
508 DLS. Points and solid line show a standard curve generated using PVA of known MW. Dashed lines
509 indicate the hydrodynamic radius of the PVA extracted from S14 (11nm) and the inferred MW (36
510 kDa). Points and error bars indicate the mean and range respectively of duplicate DLS
511 measurements.

512 Panel F shows ^1H NMR spectra used to estimate the percentage hydrolysis of the binder extracted
513 from glass fibre (S14). The upper spectrum (labelled S14) is that of the extract, with unknown
514 percentage hydrolysis and hence an unknown percentage of monomers bearing acetyl groups. The
515 five spectra below were obtained using standards prepared by proportionately mixing 80% and
516 100% hydrolysed PVA to achieve a range of acetyl group content ranging from $<0.1\%$ up to 20% (as
517 per labels to left of panel). The X-axis indicates chemical shift measured in parts per million (ppm)
518 and the Y-axis shows relative intensity.

519 **Figure 5: PVA improves vaccine thermostability on matrices**

520 A PVA-containing extract prepared from S14 was dried onto various fresh matrices, followed by
521 drying of vaccine in trehalose-sucrose buffer onto the treated matrices and untreated comparator
522 matrices. Viable virus recovery was assessed after thermochallenge as indicated for each panel. S14
523 was included as a comparator in each experiment.

524 Panel A shows loss in infectivity titre of vaccine in the absence (open circles) or presence (solid
525 circles) of PVA extracted from glass fibre (S14), as compared to -80°C stored liquid control, after one
526 week at 45 °C. Points indicate individual samples (four replicates per condition).

527 Panel B shows difference in loss in infectivity titre of vaccine loaded in polyester (leukosorb) and
528 polyamide (33100L) matrix modified by deposition of three different types of PVA prior to
529 application and drying of vaccine. Dried samples were subjected to thermochallenge for four weeks
530 at 45 °C. Points indicate individual samples (three replicates per condition).

531 Panel C shows a scanning electron microscope image of the custom-made glass fibre matrix. Scale
532 bar shows 50 µm.

533 Panel D compares loss in infectivity titre of vaccine post-thermochallenge between standard 14 and
534 custom-made glass fibre matrix modified by deposition of two different types of PVA prior to
535 application and drying of vaccine. Dried samples were subjected to thermochallenge for four weeks
536 at 45°C. Points indicate individual samples (three replicates per condition).

537

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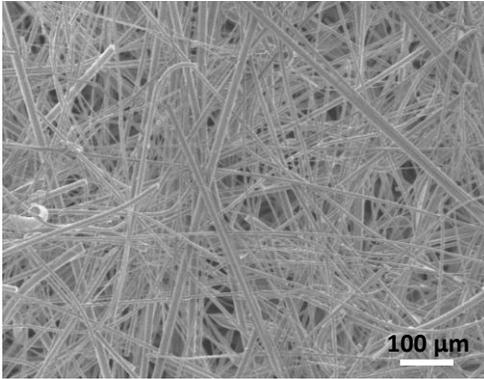
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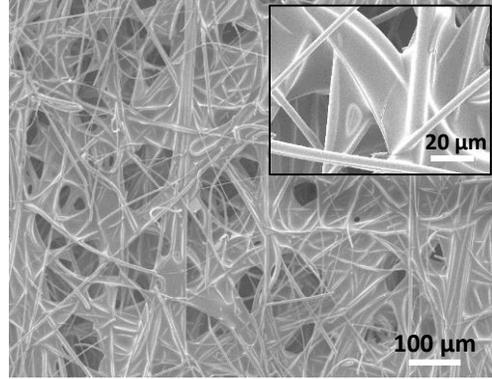
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Figure 1

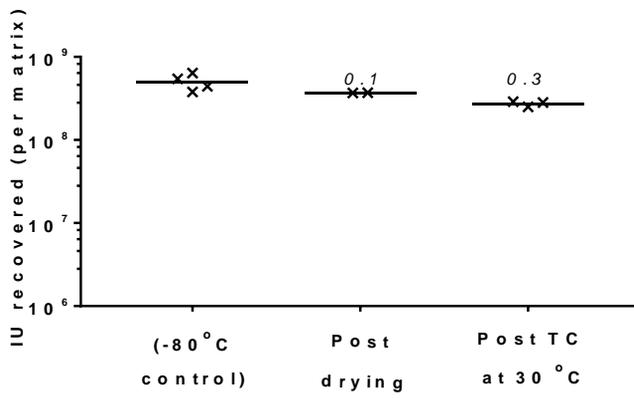
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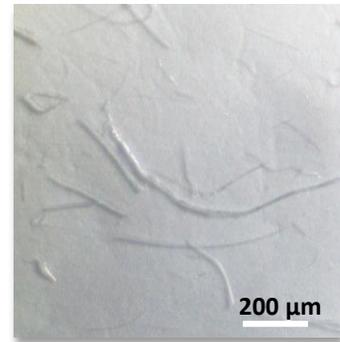


Figure 2

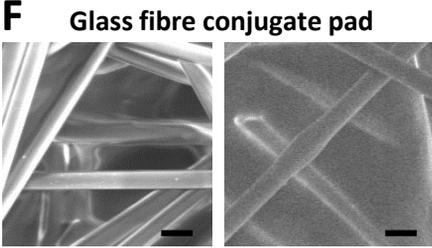
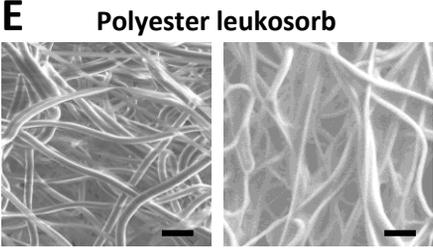
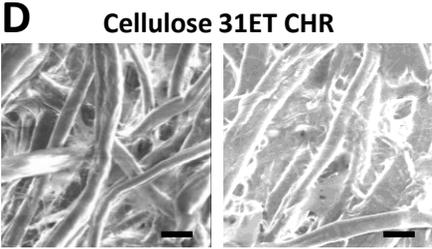
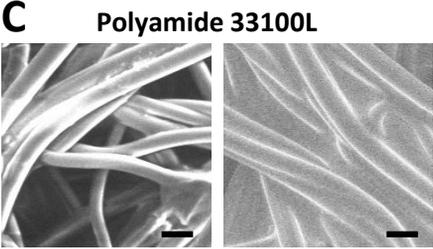
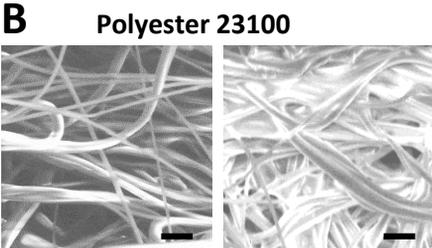
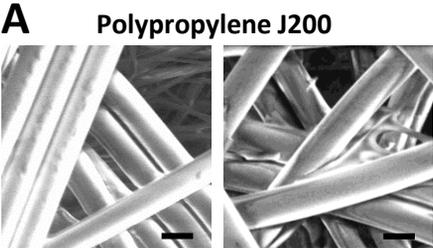
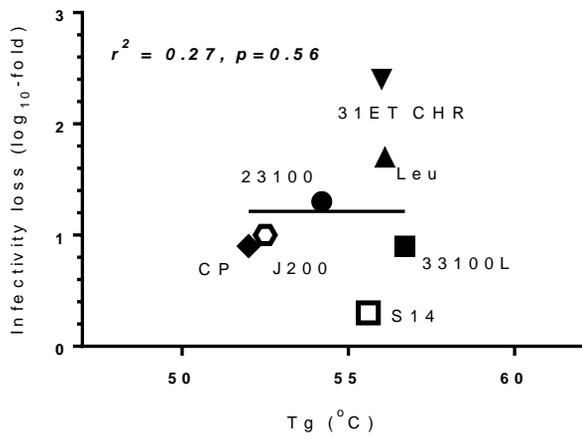
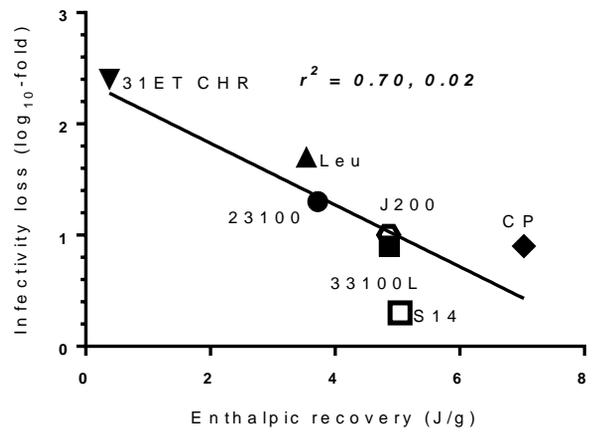


Figure 3

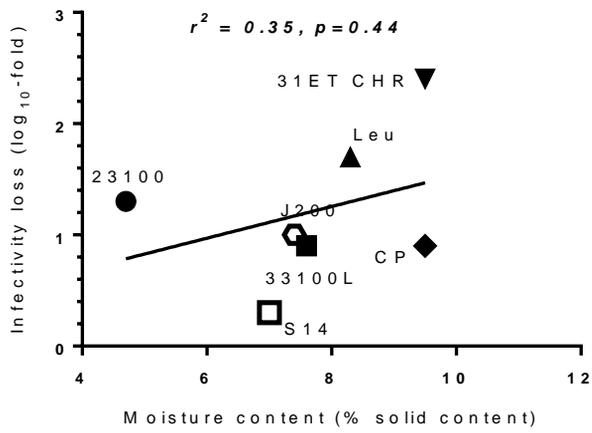
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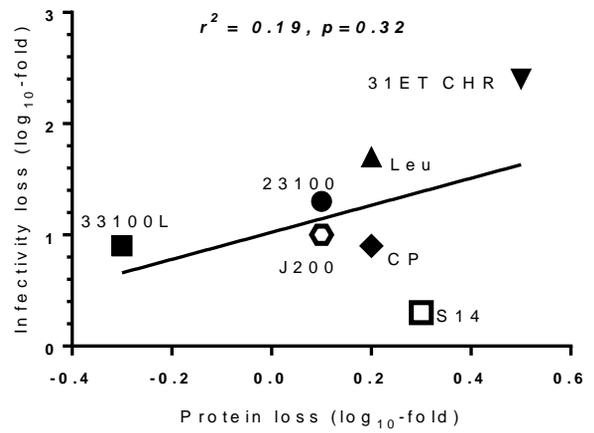


Figure 4

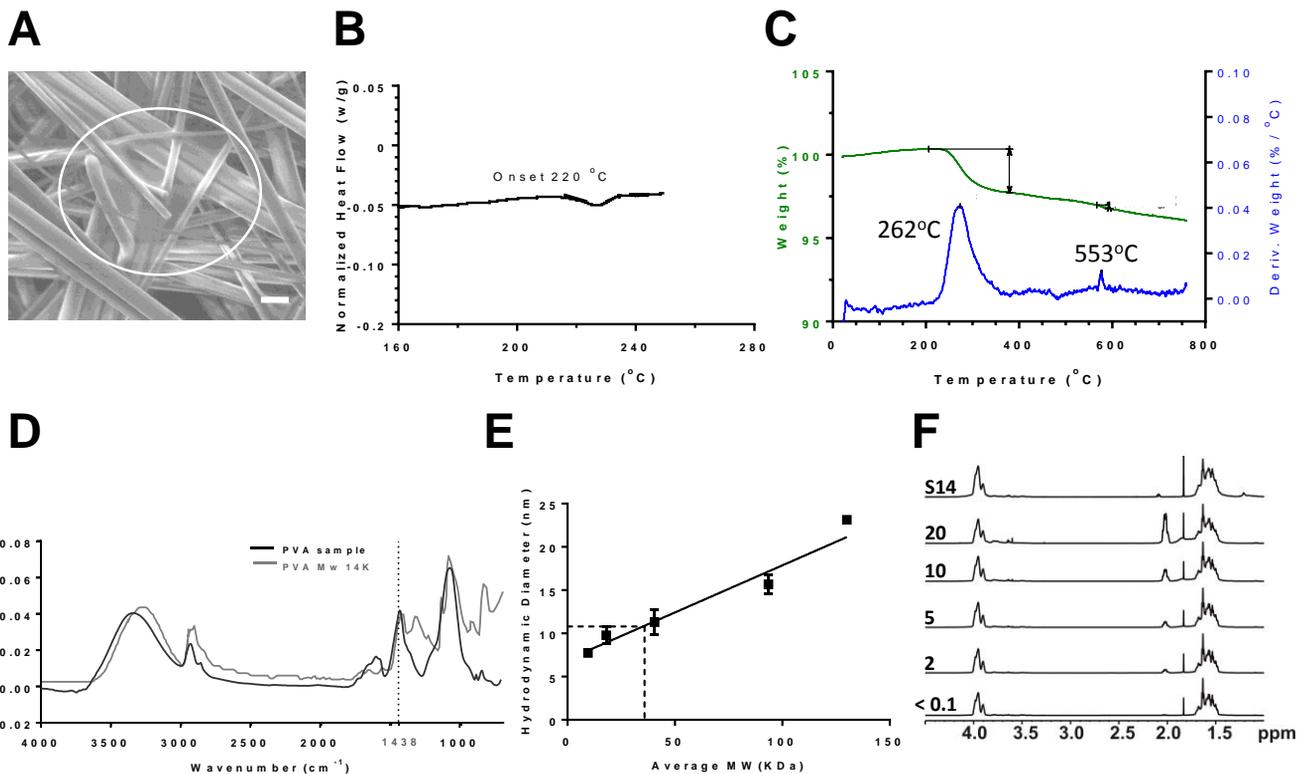


Figure 5

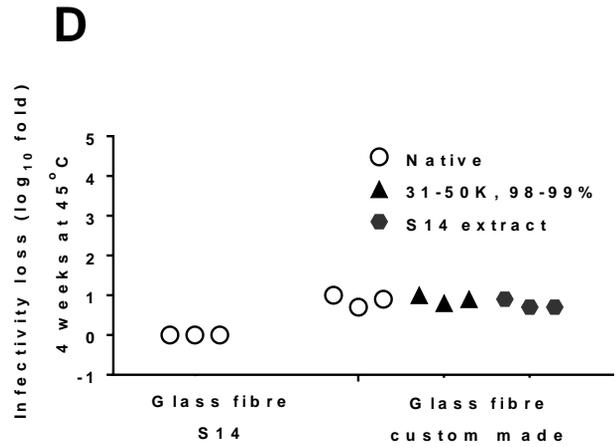
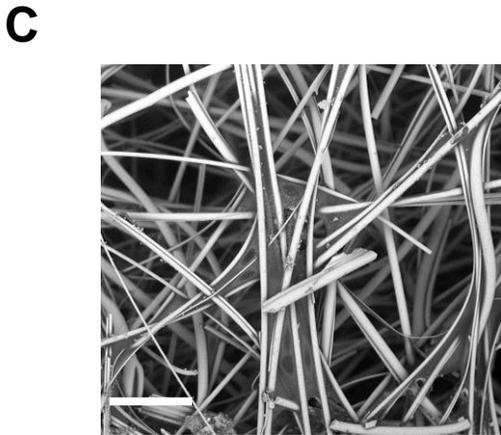
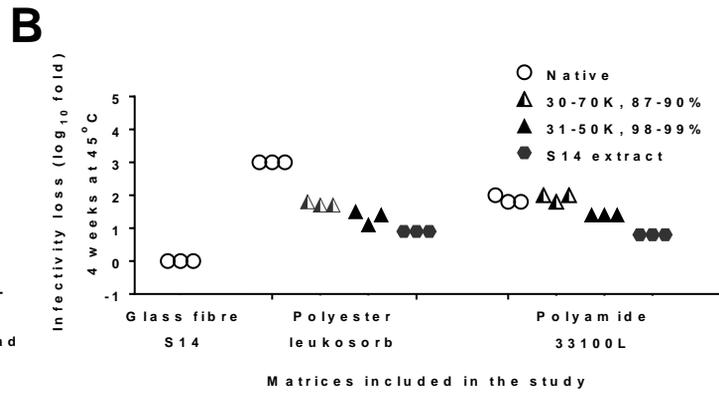
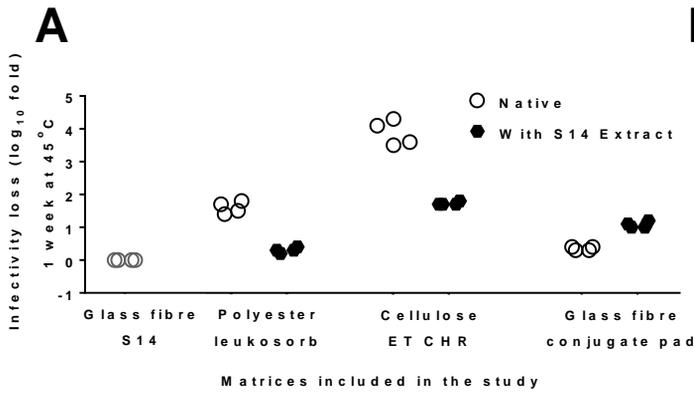


Table 1: Basic characteristics of studied matrices

Matrix name (manufacturer product reference)	Manufacturer (sub-brand if applicable)	Main material	PSS20 treatment?	Absorption >20 $\mu\text{L}/\text{cm}^2$ H ₂ O	Loading capacity ($\mu\text{L}/\text{cm}^2$)	Fibre diameter (μm) \pm SEM	Mean pore size (μm)	Thermostabilisation performance (\log_{10} -fold infectivity loss)
S 14	GE (Whatman)	Glass fibre	X	✓	55	4.2 \pm 0.3	20	0.3
J200	Pall	Polypropylene	✓	✓	36	21.7 \pm 0.4	20	1
23100	Hollingsworth - Vose	Polyester	✓	✓	43	4.4 \pm 0.4	11	1.3
33100L	Hollingsworth - Vose	Polyamide	✓	✓	36	4.8 \pm 0.4	27	0.9
Cyclopore (PC)	GE (Whatman)	Polycarbonate	✓	X	Excl.	Excl.	Excl.	Excl.
31 ET CHR	GE (Whatman)	Cellulose	X	✓	40	15.4 \pm 1.0	1.2	2.4
Leukosorb (Leu)	Pall	Polyester ^a	X	✓	40	3.5 \pm 0.2	8	1.7
Conjugate pad (CP)	Pall	Glass fibre ^b	X	✓	30	12.3 \pm 0.4	N/A	0.9
Asymmetric polysulfone (AS)	Pall	Polysulfone	✓	X	Excl.	Excl.	Excl.	Excl.

Loading capacity was measured by weight measurement of matrices before and after full impregnation in water. Fibre diameter was measured using Image J from SEM micrograph images. Mean pore size are as provided in suppliers' specification (in one case N/A denotes data not available). Thermostabilisation performance presented is loss of infectivity after vaccine drying and thermochallenge for 1 week at 45 °C. Excl. denotes matrices excluded from further analysis on basis of loading capacity.

^a Proprietary composition, but macroscopic, microscopic and FTIR spectrum appearances are consistent with polyester (data not shown); this matrix is henceforth referred to as polyester

^b Henceforth referred to as 'glass fibre (conjugate pad)' for avoidance of confusion with S14.

Table 2: Physical characteristics of the glass fibre (S14) matrix

PARAMETER (unit)	Mean	Standard deviation
Area density [g.m⁻²]	55.1	1.8
Thickness [mm]	0.5	0.04
Fibre diameter [µm]	3.4	1.8
Fibre length [mm]	4.4	1.5
Largest pore diameter [µm]	133.0	99.4
Mean flow pore diameter [µm]	22.3	0.5
Smallest pore diameter [µm]	6.4	1.5
Porosity [%]	95	
Capillary constant [cm⁵]	7.7X10 ⁻⁷	5.6X10 ⁻⁸
Thermostabilisation buffer contact angle [°]	30.9	4.9
Thermostabilisation buffer surface tension[(mN.m⁻¹)]	63.9	0.3