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1 **The Impact of Cigarette / e-Cigarette Vapour on**
2 **Simulated Pulmonary Surfactant Monolayers under Physiologically Relevant Conditions**

3
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6

7 **Abstract**

8 Deviation in pulmonary surfactant structure-function activity can impair airway patency and lead to respiratory
9 disorders. This novel study aims to evaluate the influence cigarette / e-cigarette vapour has on model
10 surfactant films located within a simulated pulmonary environment using a lung biosimulator.
11 Chromatographic analysis confirmed that nicotine levels were consistent with the sampling regimen
12 employed. On exposure to smoke vapour, Langmuir isotherms exhibited condensed character and a
13 significant reduction in maximum surface pressure was noted in all cases. Langmuir isocycles, reflective of the
14 human breathing cycle, demonstrated condensed character on smoke vapour delivery. A reduction in the
15 maximum surface pressure was clear only in the case of cigarette vapour application. The components of
16 cigarette vapour can cause oxidative damage to pulmonary surfactant and impair recycling. Neutral nicotine
17 molecules can weaken the structure of the monolayer and cause destabilisation. A protective effect was
18 evident in the case of repeated surfactant compression – relaxation cycles (i.e. the ability to reduce the surface
19 tension term was impaired less), demonstrating a likely innate biological defensive mechanism of the lung. E-
20 cigarette vapour appeared to have a reduced impact on surfactant performance, which may hold value in
21 harm reduction over the longer term.

22
23 **Key words**

24 Langmuir monolayers, pulmonary surfactant, lung biosimulator, smoking, cigarettes, e-cigarettes, gas
25 chromatography.

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39 **1. Introduction**

40

41 The primary function of the lung is to permit gaseous exchange between the body and the
42 atmosphere. The main site for such exchange is the alveolar space, which exhibits a moist and highly
43 vascularised surface of approximately 70m² [1]. The naturally occurring fluid that bathes the
44 alveolar lining is subject to considerable surface tension that can force structural collapse on
45 exhalation [2]. In order to counter this effect, and also minimise the work of breathing, a complex
46 and highly surface active mix called pulmonary surfactant is distributed at the alveolar air-liquid
47 interface [3]. The arrangement results in pulmonary surfactant presenting as the initial contacting
48 surface for aerosolised material. Prime examples of such material include respirable therapeutic
49 formulations [4] and, importantly for work presented herein, environmental toxins such as cigarette
50 / e-cigarette vapour [5 & 6].

51

52 Pulmonary surfactant is synthesised and secreted by alveolar type II cells located in the deep lung.
53 This endogenous substance exists as an insoluble film that coats the alveolar air-liquid interface [7].
54 As a result of inherent material characteristics, pulmonary surfactant is capable of reducing the
55 surface tension term to near zero values [8 & 9], which in turn facilitates alveolar stability [3]. In
56 order to achieve this, a dynamic interplay exists between the phospholipid molecules and surfactant
57 specific proteins within the naturally occurring blend. With regard to the former,
58 dipalmitoylphosphatidylcholine (DPPC) predominates and is principally responsible for the surface
59 tension lowering properties of the material [8]. As this amphiphilic molecule undergoes a gel to
60 liquid transition at 41°C, thus the ability to respread across the alveolar air-liquid interface is limited
61 during the breathing cycle [1]. Consequently, additional species are required in order to maintain
62 fluidity and support surfactant respreading. For instance, palmitoyloleoylphosphatidylglycerol
63 (POPG) facilitates effective respreading of pulmonary surfactant following compression [2].
64 Commercially available lung surfactant replacement preparations (e.g. Survanta®) are frequently
65 prescribed for the management of neonatal respiratory distress syndrome [10]. Such products are
66 often supplemented with palmitic acid (PA), which permits comparable *in vivo* respreading profiles
67 [11]. Thus, throughout this work an appropriate blend of DPPC, POPG and PA is applied to reflect
68 the key lipid fractions of pulmonary surfactant located at the alveolar air-liquid interface.

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72 A Langmuir trough may be used within the laboratory setting to represent the alveolar air-liquid
73 interface [4, 7 & 12]. Here, amphiphilic molecules arrange themselves as per the *in vivo* scenario
74 with their fatty acyl chains displaced away from the supporting aqueous subphase and the polar
75 head groups in direct contact [1]. Scope exists to control environmental parameters with the option
76 to operate at a temperature of 37°C and conduct investigations at elevated relative humidity, as per
77 the (deep) lung; this arrangement may now be investigated via the lung biosimulator [13].

78 Lateral forces may be applied to simulated pulmonary surfactant monolayers in isolation or indeed
79 succession to achieve expansion / compression cycles reflective of the human breathing pattern
80 [14]. Typical outputs from the approach include Langmuir pressure-area (π -A) isotherms and
81 isocycles, which can be applied to monitor the response of the amphiphilic material when exposed
82 to environmental stressors (i.e. cigarette smoke). For example, in 2003 Bringezu and co-workers
83 applied Langmuir monolayer technology to evaluate the effect of environmental tobacco smoke
84 (ETS) on simulated pulmonary surfactant structure-function activity [11]. The investigation utilised a
85 mixture of DPPC, POPG and PA in the ratio of 69:20:11 to maintain the lipid fraction consistent with
86 clinically used replacement pulmonary surfactant [12]. Here, the surfactant blend was applied to a
87 supporting aqueous subphase that had been previously exposed to ETS. The results from the study
88 suggested that ETS exposure impacts upon monolayer phase behaviour and morphology leading to a
89 higher minimum surface tension (i.e. reduced maximum surface pressure) and impaired lung
90 function.

91 Tobacco smoking has now become one of the most pervasive habits in modern day society [1].
92 Tobacco smoke consists of a range of chemical compounds, including aldehydes, amides, amines,
93 carboxylic acids, ketones, esters, phenols and hydrocarbons. The chemical compounds can be
94 further divided into three classes, tobacco-specific nitrosamines (TSNAs), polyaromatic hydrocarbons
95 (PAHs) and volatile organic compounds (VOCs). Compounds assigned to TSNAs, such as N'-
96 nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) comprise of
97 chemicals of known carcinogenic affect, which occur during the manufacturing, fermentation and
98 combustion of tobacco. PAHs, such as naphthalene are located in the particulate composition of
99 tobacco smoke and are produced during the incomplete combustion of the organic material.

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104 In order to minimise exposure to the toxic constituents of tobacco smoke, and hence reduce
105 associated long-term deleterious effects, the consumer now has available a range of potential
106 reduced exposure products (PREPs) to purchase [15]. One of the most recently released PREPs is the
107 e-cigarette, which is becoming increasingly popular [16]. As e-cigarettes imitate traditional
108 cigarettes, they not only deliver nicotine but also simulate the process of smoking to satisfy
109 psychological cravings. However, in contrast to traditional cigarettes, e-cigarettes do not involve
110 tobacco combustion. Here, the consumer inhales a vapour that is produced by heating a solution
111 consisting of processed nicotine extract from tobacco leaves, water, glycerine and / or propylene
112 glycol along with flavourings [17]. Potentially harmful constituents present in e-cigarette vapour
113 include carbonyl compounds, volatile organic compounds, TSNA and heavy metals [17]. All can
114 have toxic, irritating and / or carcinogenic effect on the human body [18].

115 This novel study aims to monitor the response of simulated pulmonary surfactant monolayers when
116 challenged with cigarette / e-cigarette vapour under physiologically relevant conditions (i.e. 37°C
117 and elevated relative humidity). For the first time we apply a patented technology platform to
118 quantitatively probe the influence of cigarette / e-cigarette vapour on the performance of a mixed
119 surfactant film located within an environment reflective of the (deep) lung. This work is of interest
120 because it provides a strategy by which to better understand fundamental interactions taking place
121 at a biological interface that is crucial to sustaining life. The timely work will further current
122 understanding of the health impacts associated with smoking cigarettes / e-cigarettes. Throughout
123 the piece consideration will be given to the reproducibility of nicotine presentation within the
124 sampling routine, the identification of chemical species within aerosolised samples and potential
125 mechanisms of interaction with simulated pulmonary surfactant.

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135 **2. Materials and Methods**

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137 *2.1 Materials*

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139 The surfactants DPPC (Avanti Polar Lipids, USA. Lot: 160PC-312), POPG (Avanti Polar Lipids, USA. Lot:
140 160-181PG-131) and PA (Sigma-Aldrich, UK. Lot: PO500) were of analytical grade and used as
141 supplied. Chloroform (CHCl₃) (Sigma-Aldrich, UK) of analytical grade ($\geq 99.9\%$) was employed to
142 clean contacting surfaces and as the spreading solvent. Methanol (HPLC Grade, Sigma-Aldrich,
143 34860, Lot: STBF7002V) was employed as the solvent during smoke analysis via gas chromatography.
144 Ultrapure water (Purite, UK), demonstrating a resistivity of 18.M Ω cm, was used both during cleaning
145 procedures and as the Langmuir monolayer aqueous subphase. Marlboro Gold cigarettes along with
146 Blu Classic (first generation) and Eleaf iStick 50W, with Eleaf GS Air Tank atomiser (3rd generation) e-
147 cigarettes were purchased through a retail sources. The strength of the e-cigarette refills was
148 represented by the amount of nicotine (i.e. mg) per 1ml of the liquid solution. The cartridges used
149 with the first generation device contained 18mg of nicotine per unit. The batteries of each device
150 were fully charged before each test to facilitate reproducible data collection.

151

152 *2.2 Methods*

153

154 *2.2.1 Langmuir Monolayer Preparation*

155

156 Surfactant monolayers were produced using a Langmuir trough (Model 102M, Nima Technology,
157 UK). Surfactant free tissues (Kimtech Science, Kimberley-Clark Professional, 75512, UK) were soaked
158 in chloroform and used to clean all contacting glassware and surfaces. Background tests to monitor
159 surface pressure in the absence of surfactant material were performed to ensure trough cleanliness,
160 which was accepted at surface pressures of 0.4mN/m or less on complete barrier compression. A
161 spreading solution composed of DPPC, POPG and PA in the ratio 69:20:11 was produced to reflect
162 appropriate lipid fractions at the alveolar air-lipid interface by dissolving the surfactant material in
163 chloroform to a concentration of 1 mg/ml. In total, 10 μ l of this solution was delivered to the surface
164 of the ultrapure water subphase (50ml) at pH 7 by dropwise addition using a Hamilton microsyringe.
165 The volume of 10 μ l was chosen so as to achieve a steady transition from the gaseous phase through
166 to condensed phases on barrier compression and prevent saturation of the π -A isotherms / isocycles
167 at the solid phase point.

168

169 A period of 10 minutes was allowed to allow chloroform evaporation and surfactant spreading over
170 the 70cm² area. The polytetrafluoroethylene trough barriers were programmed to move to the
171 centre of the trough at a rate of 25cm²/min. Plots of surface pressure vs. percentage trough area for
172 the surfactant system at 37°C and elevated humidity (e.g. 80% RH) were collected using a Wilhelmy
173 plate, formed from Whatman 44 filter paper, at the centre of the compartment.

174

175 2.2.2 Cigarette / e-cigarette Vapour Generation

176

177 The vapour collection regimen involved taking 2 puffs from the cigarettes / e-cigarettes of 50ml total
178 volume, over a 4-second puff duration with a 30-second puff interval [19]. The vapour was collected
179 in a 250ml quick fit round bottom flask with 3 outlets. Each cigarette / e-cigarette was connected to
180 a Teflon mouthpiece that was linked to one of the outlets of the round bottom flask using
181 appropriate tubing. The second outlet, of the same size was connected to a 500ml separating funnel
182 and the third outlet was closed with stopper to produce an airtight system. The experimental
183 arrangement for smoke collection is presented in Figure 1.

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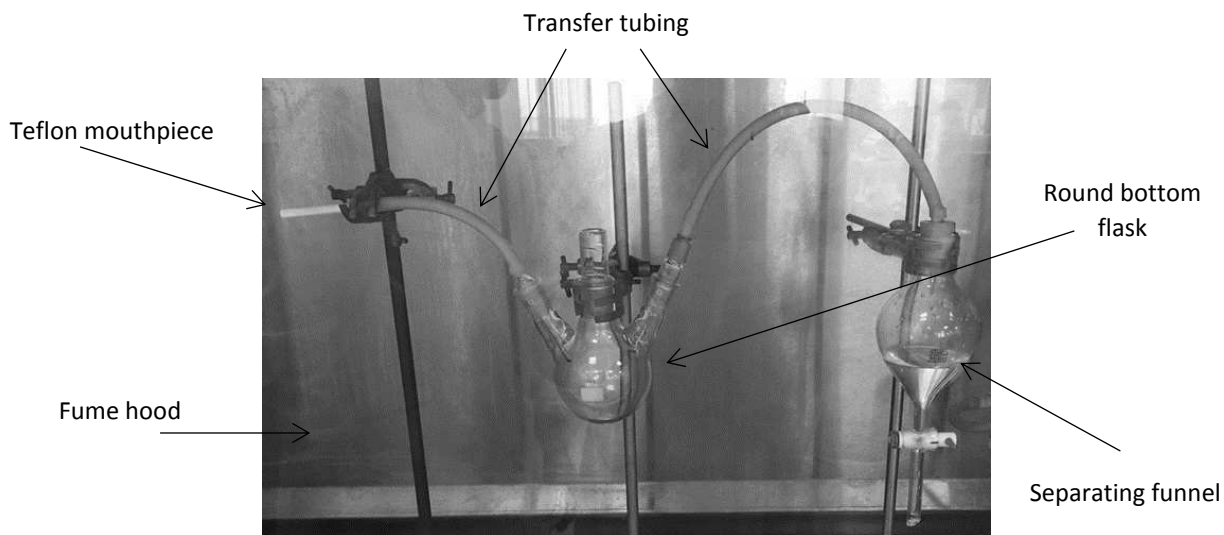
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195 **Figure 1.** The arrangement applied to collect smoke vapour aliquots.

197 Before each cigarette / e-cigarette was activated, a total of 100ml of water was poured into the
198 separating funnel (i.e. equivalent to 2 puffs). On activation, smoke vapour was collected in the
199 round bottom flask by withdrawing the 50ml of water from the funnel, with the next puff drawn
200 after 30 seconds [19]. Once the second vapour aliquot was obtained, the round bottom flask
201 containing smoke was disconnected from the separating funnel and mouthpiece and the two outlets
202 are closed with stoppers to hold the smoke inside the flask.

203

204 2.2.3 Nicotine Quantification / Smoke Component Determination

205

206 Following the collection of each vapour sample, a total of 2ml of methanol was added to the round
207 bottom flask to solubilise the aerosolised material. Each sample was then filtered with a 0.45µm
208 syringe filter into a glass vial insert. Analysis of nicotine standards and smoke extracts was carried
209 out on an Agilent 7980GC with flame ionisation detection (FID). The analytical column selected was
210 an Agilent J&W DB-1 (30m x 0.250mm x 0.50µm), with a column temperature of 160°C (isocratic).
211 The injection type was 1µl split (10:1) (20ml/min 250°C), with nitrogen selected as the carrier gas
212 and the flame ionisation detector temperature programmed at 250°C. Nicotine standards ranging
213 from 0.0078 - 1mg/ml were constructed for nicotine quantification of the vapour extracts.
214 Standards displayed excellent linearity with R² values >0.999. The analysis of 5 replicate smoke
215 samples per cigarette/e cigarette was undertaken.

216 Evaluation of vapour components was determined using an Agilent 6980GC with 5975MS detection.
217 The column was an Agilent J&W HP5-MSUI (30m x 0.250mm x 0.25µm) with split (10:1) injection of
218 1µl. The oven temperature were: 50°C for 5mins, 20°C/min to 255°C held for 1 min, 20°C/min to
219 300°C held for 5 mins. The mass spectrometer was run in full scan mode from 40-500 AMU. Mass
220 spectra for recorded peaks were further evaluated using the NIST database (MS search programme
221 Version 2.0, NIST, MSS Ltd., Manchester, England).

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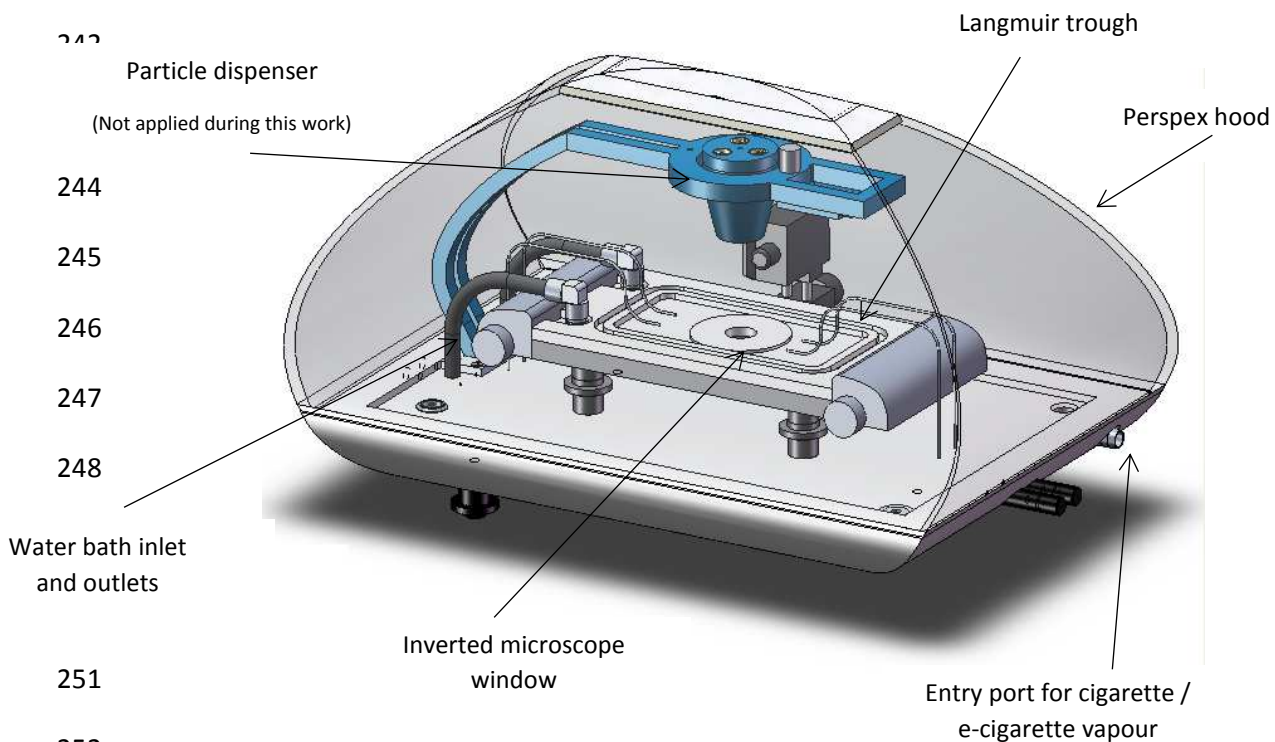
232 2.2.4 Vapour Addition to Simulated Pulmonary Surfactant Monolayers

233

234 In order to assess the impact of smoke vapour on simulated pulmonary surfactant monolayers under
235 physiologically relevant conditions, the aerosolised material was transferred from the round
236 bottomed flask to the enclosed lung biosimulator [13], as detailed in Figure 2, using compressed air.
237 Initially, baseline data was collected in the absence of cigarette / e-cigarette vapour. Subsequently,
238 the smoke vapour acquired from either the cigarettes or e-cigarettes was delivered to the test zone.
239 In each case, a period of 10 minutes was allowed for interaction between each species under
240 consideration.

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254 **Figure 2.** A schematic detailing the lung biosimulator.

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259 To obtain Langmuir isotherms, a single compression was applied towards the centre of the trough at
260 a rate of 25cm²/min. This relatively slow speed was chosen to closely observe the direct impact of
261 cigarette / e-cigarette vapour on both the physical state of the simulated pulmonary surfactant plus
262 compression performance. With respect to Langmuir isocycle tests, a total of 14 compression-
263 expansion cycles were undertaken at a speed of 100cm²/min. This faster compression speed is more
264 representative of the human breathing cycle and provides an insight into system dynamics on
265 exposure to cigarette / e-cigarette vapour. In this case, the first 4 cycles were used to condition the
266 monolayer such that the equilibrium position was attained. This approach enabled a clearer
267 depiction of the influence of the cigarette / e-cigarette vapour on the simulated pulmonary
268 surfactant monolayer. All Langmuir isotherm tests were repeated five times, whilst Langmuir
269 isocycles were repeated three times and averaged data was used to generate the plots presented
270 herein. On test completion, the remaining vapour was removed from the lung biosimulator by
271 directing through a tube to a nearby fume hood using compressed air.

272

273 2.2.5 The Compressibility of Langmuir Monolayers

274

275 The compressibility term relating to a Langmuir monolayer refers to the ability of the material to
276 lower the surface tension at the air-liquid interface with minimal change in surface area [20].
277 Surfactant films should ideally have a low compressibility value such that gaseous exchange can take
278 place over a large surface area [21]. The lower the compressibility term, the more rigid the
279 surfactant film is (i.e. the material is of low elasticity), with the opposite being true [22 & 23]. The
280 parameter is calculated as detailed in Equation 1.

281

$$282 \text{ Compressibility} = \frac{1}{A} \times \frac{1}{m}$$

283

284 **Equation 1.** *Simulated pulmonary surfactant compressibility determination.*

285

286 Where A represents the relative surface area and m the slope of the isotherm. Here, 'm' was
287 calculated via $m = \frac{y_2 - y_1}{x_2 - x_1}$ over the surface pressure range of 10-30mN/m, whereby 'y' and 'x' values
288 characterise surface pressure and area values, respectively [20].

289 **3. Results & Discussion**

290

291 *3.1 Chemical Analysis of Smoke Vapour and Potential Impact on the Body*

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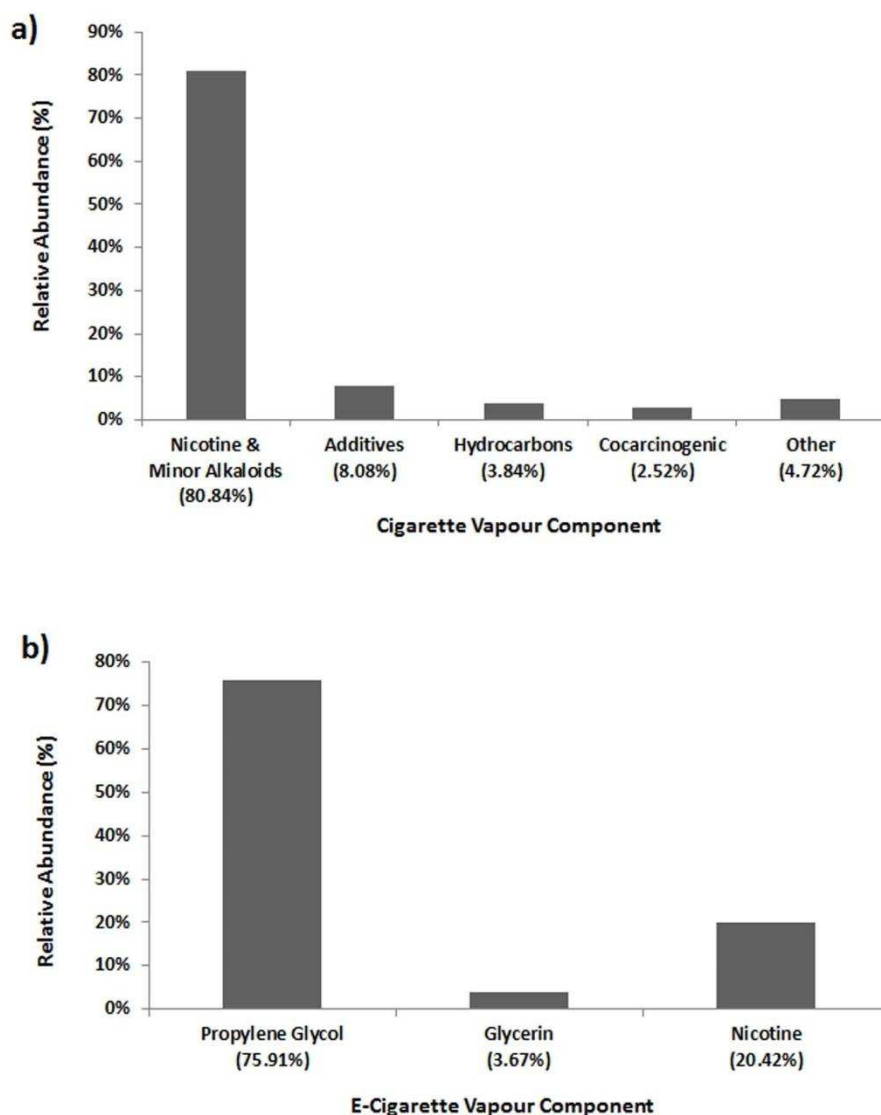
293 Cigarette smoke contains thousands of chemical components, some of which are naturally occurring
294 within the tobacco plant whilst others are added as additives during manufacture [24]. The nicotine
295 component of the Marlboro Gold cigarette vapour tested herein was 0.043mg/ml \pm 0.009, the
296 quantity of this compound corresponded to that stated by the manufacturing company. The 1st
297 generation e-cigarette vapour produced a mean nicotine concentration of 0.048 mg/ml \pm 0.006, with
298 the 3rd generation e-cigarette vapour producing a value of 0.035 mg/ml \pm 0.003. The data
299 demonstrated good reproducibility through all cigarette types.

300

301 *3.2 Gas Chromatography / Mass Spectroscopy Data*

302

303 GC-MS analysis of the cigarette / e-cigarette vapour component composition is illustrated in Figure 3a
304 and Figure 3b.



305

306 **Figure 3.** The principal components of cigarette vapour as determined by GC-MS. (a) cigarette vapour; (b) e-
 307 cigarette vapour.

308

309 The analysis confirms that nicotine and the related minor alkaloid components are the most abundant
 310 compounds within the cigarette vapour. In addition, the vapour sample demonstrated a proportion
 311 of additive compounds. The compounds representing the 'other' section included amines, and smoke
 312 related vapours, such as toluene. With reference to the composition data relating to both the 1st and
 313 3rd generation e- cigarette vapour, it is apparent that nicotine is present, but it is not the major
 314 component. The addition of propylene glycol and glycerin to the e-cigarette formulations accounts
 315 for a large proportion of the compounds present (i.e. >75% of the total composition) [18].

316 Toluene and xylene were detected within the cigarette vapour extract by the GC-MS element of this
317 investigation. Exposure to the former can be detrimental to white blood cell function and this can in
318 turn pre-dispose to respiratory tract infections [25]. Furthermore, exposure to xylene at levels
319 greater than 200 ppm can irritate the lungs leading to acute shortness of breath accompanied by
320 chest pain [26].

321 In terms of the e-cigarette vapour, this route of nicotine administration to the body may be
322 considered less harmful than the more natural, counterpart products. With regard to this system of
323 nicotine delivery, during 2011 Trehy and co-workers documented that the composition of refill
324 products varies considerably as a result it is difficult to fully evaluate the hazards related to
325 electronic cigarette usage [27]. The content of the aerosol generated from e-cigarette is highly
326 variable, not only among different products but also within different samples of the same e-liquids
327 [16, 17, 27, 28, 29 & 30]. Therefore, we suggest that further work is required to better understand
328 the impact of the spectrum of e-cigarette products may have on pulmonary function.

329 During this work we have carefully replicated the main stages of cigarette / e-cigarette use via
330 reference to a typical puffing regimen [19] and applied the acquired vapour to a test zone housing a
331 model pulmonary surfactant system representative of typical *in vivo* lipid fractions under
332 physiologically relevant conditions [11]. The accepted mechanism of action for pulmonary
333 surfactant, and model mixtures thereof, revolves around the unsaturated lipid fraction (e.g. POPG)
334 forming a fluid-like liquid-expanded matrix to separate phases rich in condensed saturated lipids
335 (e.g. DPPC) [1 & 31]. The delicate coexistence between each phase at the alveolar air-liquid
336 interface is essential for effective surfactant function (i.e. to regulate surface viscosity and lower
337 surface tension) [11, 14 & 31]. Clearly, any disruption to the synergy between the liquid-expanded
338 and liquid-condensed phases forming the surfactant film can have a detrimental impact on gross
339 lung function [1 & 21]. Within the laboratory setting, deviation in recorded Langmuir pressure-area
340 isotherms and / or isocycles provides direct evidence of changes to overall surfactant performance.

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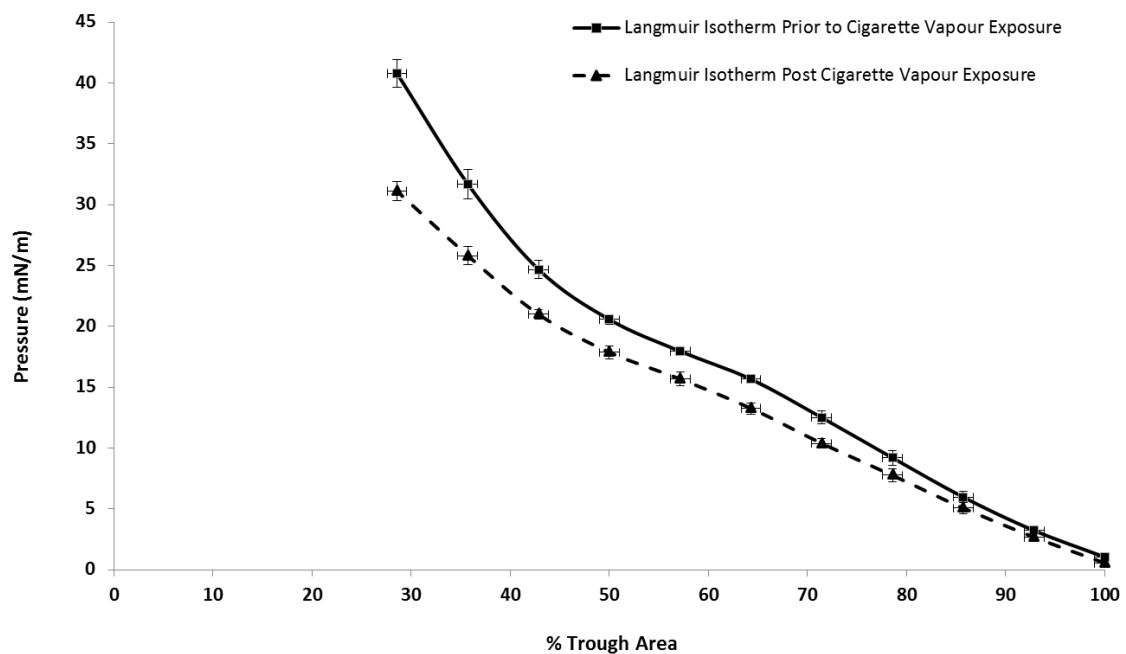
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347 3.3 Langmuir Pressure – Area Isotherms

348

349 Langmuir pressure-area isotherms were acquired for the simulated pulmonary surfactant systems
350 when exposed to either cigarette or e-cigarette vapour under conditions reflective of the (deep)
351 lung; relevant data are presented in Figures 4 and 5, respectively. All systems exhibit two-
352 dimensional phase changes over the course of compression; movement through the gaseous,
353 expanded and condensed phases is confirmed on gradient change from right to left. Here, the
354 compressibility parameter was considered with the slope of the trace used as a marker for the
355 compressibility of the two-dimensional film; where the steeper the slope, the harder it is to
356 compress the surfactant monolayer [32].



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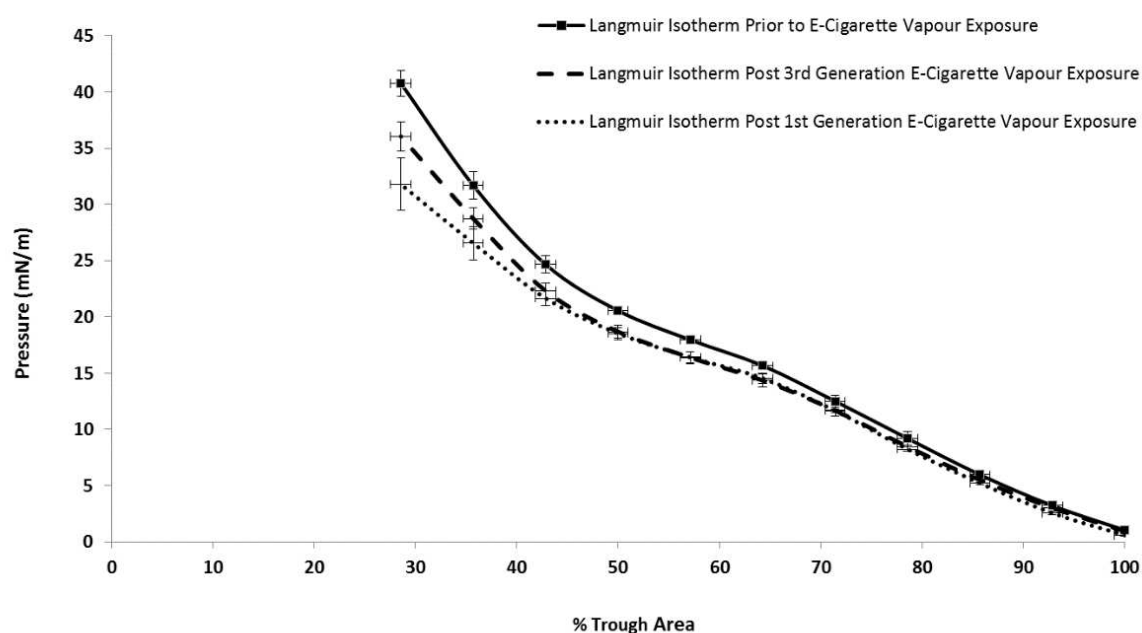
359 **Figure 4.** A Langmuir pressure-area isotherm detailing the response of a simulated pulmonary surfactant
360 monolayer to cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated
361 relative humidity. Averaged data of 5 replicates presented with standard error of the mean displayed.

362

363 On inspection of the data presented in Figure 4, it is clear that the administration of cigarette vapour
364 to the test zone did influence simulated pulmonary surfactant structure-function activity. Here, the
365 ability to attain low surface tension values at any given relative area is reduced and there is an
366 increase in the ease of compression under physiologically relevant conditions (i.e. the monolayer is
367 more compressible).

368 In the case of the model surfactant system studied herein, the highest surface pressure recorded in
369 the absence of cigarette smoke was 41mN/m. This value was as a direct result of applying 10 μ l of
370 the surfactant spreading solution (1mg/ml) to the supporting aqueous subphase, which was deemed
371 appropriate to achieve smooth lipid phase transitions during compression and prevent solid phase
372 saturation at minimal trough areas. If a larger spreading solution volume were to be applied to the
373 aqueous subphase then the maximum surface pressure would rise (e.g. attain a value of
374 approximately 70mN/m). On application of cigarette vapour, the value of 41mN/m diminished to
375 32mN/m. Hence, the capacity to lower the surface tension at full monolayer compression was
376 reduced by 22%. In addition, exposure of cigarette vapour resulted in the monolayer exhibiting a
377 condensed character (i.e. being transposed to the left of the baseline plot). Comparable trends, as
378 those noted here, would be anticipated at higher surface pressure values (e.g. 70mN/m) [11].

379 A similar response was noted when 1st and 3rd generation e-cigarette vapour was delivered to the
380 test zone. Once again the baseline plot for our system exhibited a maximum surface pressure of
381 41mN/m (i.e. due to the application of 10 μ l of material) with reduction in the term evident on
382 exposure to 1st generation and 3rd generation e-cigarette vapour; namely 32mN/m and 36mN/m,
383 respectively. It is interesting to note that on delivery of the 1st generation e-cigarette vapour an
384 identical reduction in the surface pressure term of 22% was noted. This deviation was less in the
385 case of the 3rd generation product, namely a 12% reduction. The presence of e-cigarette vapour led
386 to a reduction in the maximum surface pressure from the baseline data, this finding is statistically
387 significant due to the absence of overlap in the presented standard error of the mean bars.
388 Furthermore, as previously noted exposure to e-cigarette vapour caused a clear decrease in surface
389 pressure at any corresponding area.
390



391

392 **Figure 5.** Langmuir pressure-area isotherm data outlining the response of a simulated pulmonary surfactant
 393 monolayer to e-cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated
 394 relative humidity. Averaged data of 5 replicates presented with standard error of the mean displayed.

395

396 Similar responses to those outlined above have been noted within the literature [11]. All data
 397 presented within this piece are reflected of the *in vivo* situation where smoke vapour would interact
 398 with pulmonary surfactant via a ‘top-down’ approach. In this instance, the hydrocarbon chains of
 399 the phospholipid molecules were primarily exposed to those chemicals within the smoke aliquots.
 400 Therefore, this work considers real-world interfacial interactions that can potentially compromise
 401 the biological function of the lung. Furthermore, in support of our findings Kannisto and Yhteiskoulu
 402 reported functional changes in the lipid fraction of pulmonary surfactant as a result of phospholipid
 403 degradation and / or the penetration of nicotine molecules into the two-dimensional film during
 404 their 2006 study [33].

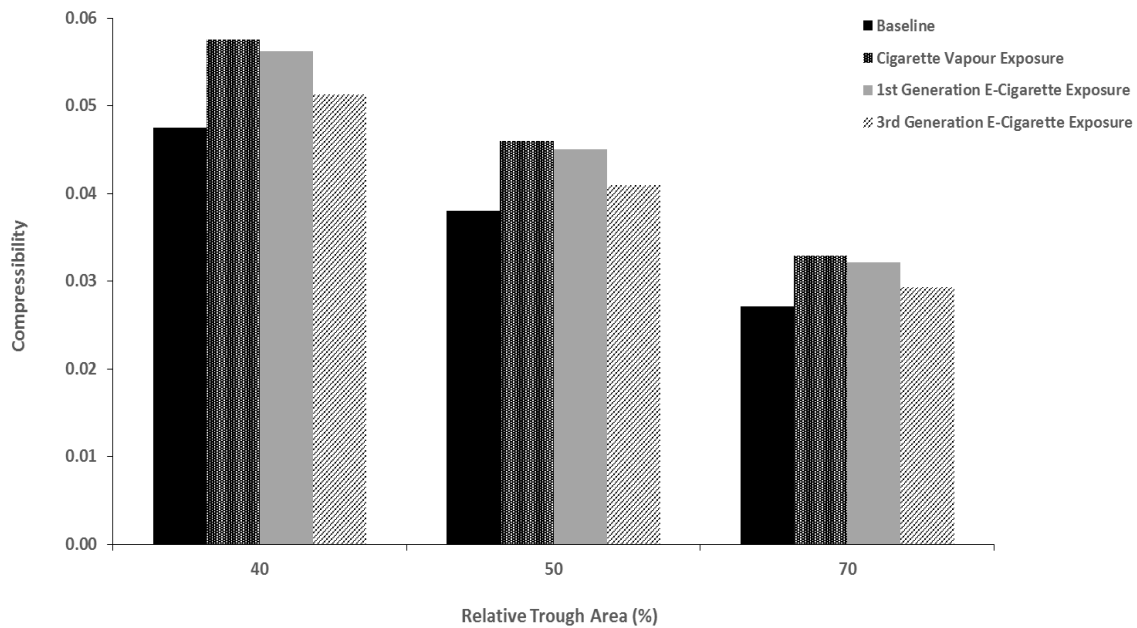
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406 3.3.1 Langmuir Isotherm Compressibility Analysis

407

408 In order to quantify the impact of cigarette / e-cigarette vapour had on simulated pulmonary
 409 surfactant compressibility Equation 1 was applied. Here, the slope of the Langmuir pressure-area
 410 isotherm was considered along the liquid-expanded to liquid-condensed transition. That is to say
 411 between the surface pressures of 10mN/m to 30mN/m at the specific relative trough areas of 40%,
 412 50% and 70%. Compressibility data for each system is presented in Figure 6.

413



414

415 **Figure 6.** *The compressibility of simulated pulmonary surfactant monolayers at pre-defined relative trough*
416 *areas in the absence and presence of cigarette / e-cigarette vapour. In all cases of single monolayer*
417 *compression (i.e. Langmuir isotherms), the delivery of such vapour to the test zone increased the*
418 *compressibility term.*

419

420 On exposure to cigarette / e-cigarette vapour, the compressibility term increased in all cases.
421 Greater compressibility values indicate that the surfactant film becomes less rigid in nature and
422 more elastic (i.e. easier to compress when compared to the baseline). This effect is more
423 pronounced in the case of exposure to cigarette vapour. The impact on monolayer compressibility is
424 limited in the case of the 3rd generation e-cigarette.

425 Although the use of Langmuir isotherms is not representative of the human breathing cycle, which is
426 dynamic in nature, we believe that the information obtained from this largely static system can
427 provide insight into the way in which environmental toxins (e.g. cigarette / e-cigarette vapour) can
428 influence individual molecular species that are in the main fully exposed at the alveolar air-liquid
429 interface (i.e. when in the gaseous phase). Here, we liken this situation to a lone soldier under
430 attack from an opposing force.

431

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435 In all cases, exposure to cigarette / e-cigarette vapour resulted in the simulated pulmonary
436 surfactant monolayer exhibiting a condensed character. Consequently, the ability to reduce the
437 surface tension term was impaired across all relative trough areas during compression to the centre
438 of the compartment. In addition, there was an apparent increase in monolayer compressibility.
439 Clearly, exposure to vapour from all platforms had a detrimental impact on simulated pulmonary
440 surfactant performance with exposure to cigarette vapour and the 1st generation e-cigarette vapour
441 being the most significant. There are a number of reasons to explain the notable trend in the data
442 sets presented herein. A previously reported aspect involves a reduction in phospholipid content
443 within the surfactant film due to exposure to the chemical constituents of smoke vapour (e.g. free
444 radicals and oxidising agents) [11]. Importantly, we believe that a key mechanism of surfactant film
445 degradation lies in the ability of neutral nicotine molecules within smoke vapour to penetrate in-
446 between the relatively exposed phospholipid polar head groups of the surfactant film. On
447 inhalation, nicotine in the unionised form is able to enter the body and can readily pass across
448 membrane structures as opposed to protonated nicotine [34]. As such, the tobacco industry
449 typically designs cigarettes to have a large proportion of unprotonated nicotine for inhalation to
450 enhance lung deposition and delivery to the brain [35]. Consequently, when the surfactant film is in
451 the uncompressed state (i.e. with the individual surfactant molecules decidedly exposed for
452 interaction) neutral nicotine could potentially weaken intermolecular van der Waals forces and
453 cause structural destabilisation, which will ultimately increase the compressibility of the material
454 (i.e. cause it to be less rigid) [33].

455 Tobacco-specific nitrosamines can also have a detrimental impact on the mechanical properties of
456 surfactant monolayers (i.e. by degrading individual phospholipid molecules) [36]. For example, NNN
457 and NNK are primary carcinogenic tobacco-specific nitrosamines that are present in cigarette smoke
458 [37]. Upon interaction with a surfactant film, these agents enhance phospholipid hydrolysis and
459 subsequently reduce content within the alveolar space; an accompanied increase in
460 lysophospholipid is also noted [28]. Within the body, lysophospholipids are formed as a result of
461 phospholipase A2 stereoselective hydrolysis of the ester linkage of phospholipids to release fatty
462 acids and lysophospholipids [38]. The lysophospholipids produced also have a direct detergent-like
463 effect on the surfactant leading to impaired surface activity and consequently lead to a reduction in
464 rigidity across the two-dimensional plane [21].

465

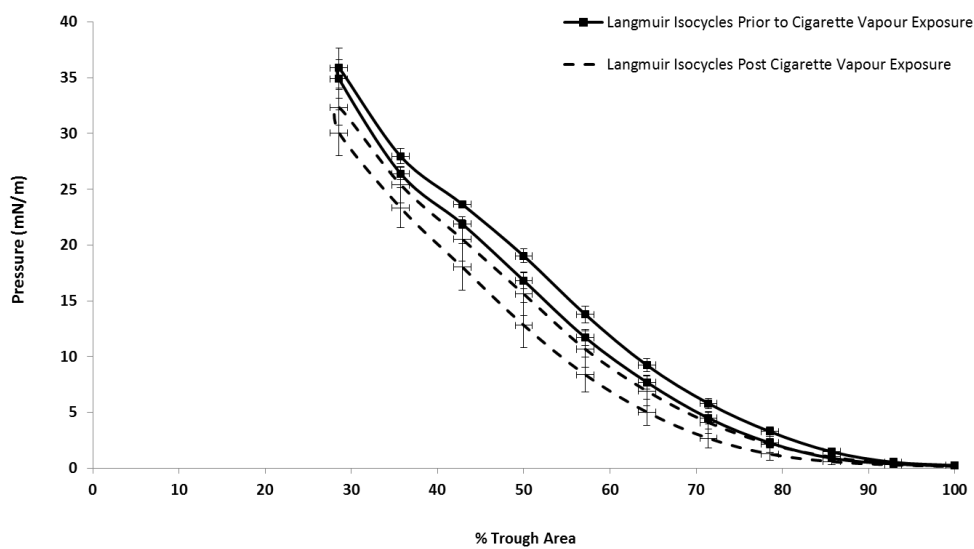
466

467

468 3.4 Langmuir Pressure – Area Isocycles

469

470 Langmuir pressure-area isocycles were also recorded for each system under conditions reflective of
471 the *in vivo* scenario such that the impact of smoke vapour on surfactant dynamics could be assessed;
472 representative plots are presented in Figures 7, 8 and 9. Again, the presence of cigarette / e-
473 cigarette vapour within the test zone did impact simulated pulmonary surfactant function. In each
474 case, the surfactant film exhibits a condensed character and the ability to lower the surface tension
475 at all stages throughout compression is weakened.



476
477

478 **Figure 7.** Langmuir pressure-area isocycle data relating to the response of a simulated pulmonary surfactant
479 monolayer to cigarette vapour addition under physiologically relevant conditions, namely 37°C and elevated
480 relative humidity. Averaged data of 3 replicates presented with standard error of the mean displayed. Where,
481 each replicate consists of 10 compression-expansion cycles at a barrier speed of 100cm² / min.

482

483 With regard to the baseline systems (i.e. Langmuir isocycles in the absence of cigarette / e-cigarette
484 vapour), the maximum recorded surface pressure was 36mN/m during this work on addition of 10µl
485 spreading solution to the surface of the supporting aqueous subphase. This value is comparable to
486 that previously observed for the Langmuir isotherm element of this study, with the slight reduction
487 due to monolayer pre-conditioning (i.e. the execution of 4 compression – expansion cycles) to attain
488 the equilibrium state.

489

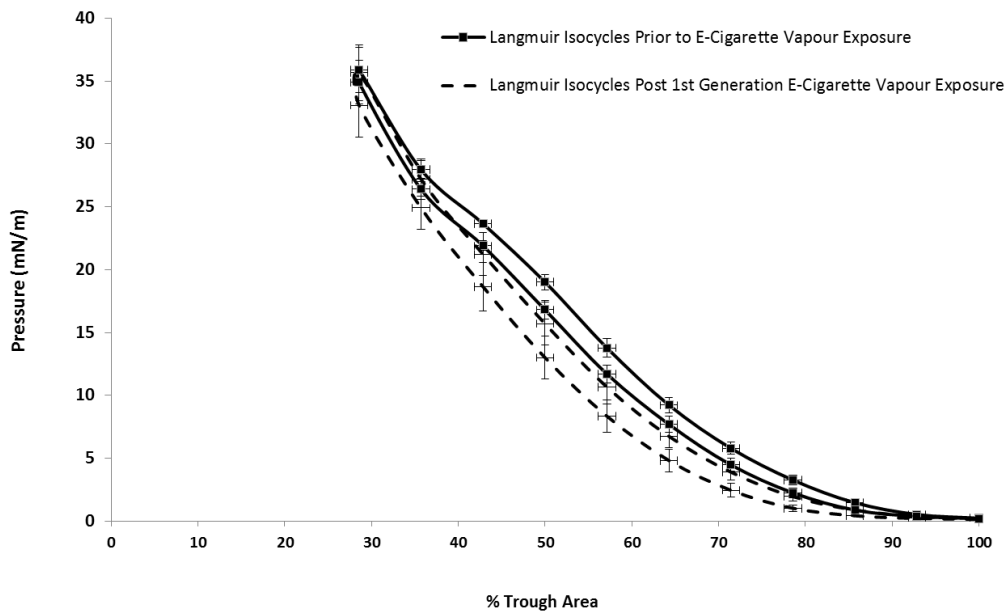
490

491 Following exposure to cigarette vapour, the ability of the simulated pulmonary surfactant film to
492 reduce the surface tension term was impaired at all relative trough areas. The result may be
493 ascribed to a reduction in the total phospholipid / lipid content of the surfactant film [14 & 21].
494 Moreover, if the gradient of the trace between the surface pressures of 10mN/m and 30mN/m is
495 considered, it is apparent that the surfactant film exposed to the cigarette vapour is less
496 compressible (i.e. harder to compress) when compared to the baseline isotherm. Thus, the data
497 indicate that exposure to cigarette vapour increases the work required to compress the simulated
498 pulmonary surfactant monolayer to the minimum trough area.

499 On expansion, the simulated pulmonary surfactant monolayer exposed to cigarette smoke followed
500 a similar pattern to that of the baseline system. The result confirms that the material is able to
501 respread after exposure to smoke vapour. Furthermore, the apparent hysteresis between
502 compression and expansion cycles was constant. Interestingly, the difference in collapse pressure
503 before and after exposure to smoke was less significant compared to the single compression
504 isotherm presented in Figure 4; in this case only an 11% reduction was calculated for the term. We
505 attribute this result to a 'protective mechanism' on dynamic monolayer compression – expansion
506 cycling and suggest that the lipid peroxidation effects contribute to the chemical degradation of the
507 POPG molecule that is primarily responsible for maintaining the fluidity of the surfactant film.

508 Following exposure to e-cigarette vapour, the simulated pulmonary surfactant monolayers were not
509 significantly degraded and once again displayed condensed character as illustrated in Figures 8 and
510 9. Here, the ability to lower the surface tension term at all relative areas was reduced, as previously
511 noted in the case of the cigarette vapour addition. In contrast to the previous system, the data
512 confirm that the maximum surface pressure of 36mN/m is attained subsequent to e-cigarette
513 vapour exposure. Thus, there is limited impact on attaining the maximum surface pressure value.

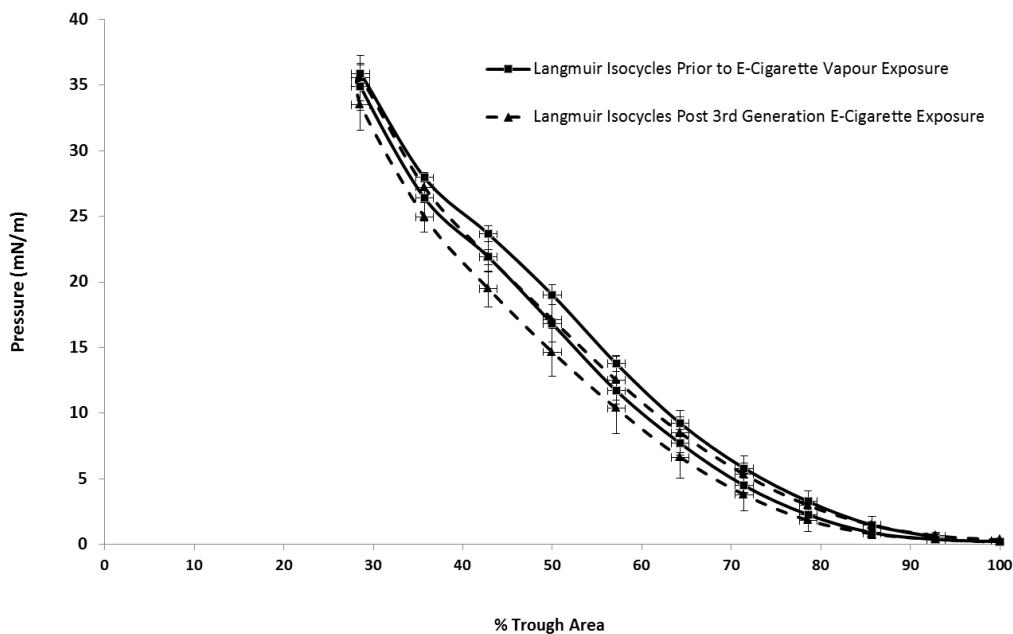
514



515

516 **Figure 8.** Langmuir pressure-area isocycle data relating to the response of a simulated pulmonary surfactant
 517 monolayer to 1st generation e-cigarette vapour addition under physiologically relevant conditions, namely 37°C
 518 and elevated relative humidity. Averaged data of 3 replicates presented with standard error of the mean
 519 displayed. Where, each replicate consists of 10 compression-expansion cycles at a barrier speed of 100cm² /
 520 min.

521



522

523 **Figure 9.** Langmuir pressure-area isocycle data relating to the response of a simulated pulmonary surfactant
 524 monolayer to 3rd generation e-cigarette vapour addition under physiologically relevant conditions, namely 37°C
 525 and elevated relative humidity. Averaged data of 3 replicates presented with standard error of the mean
 526 displayed.

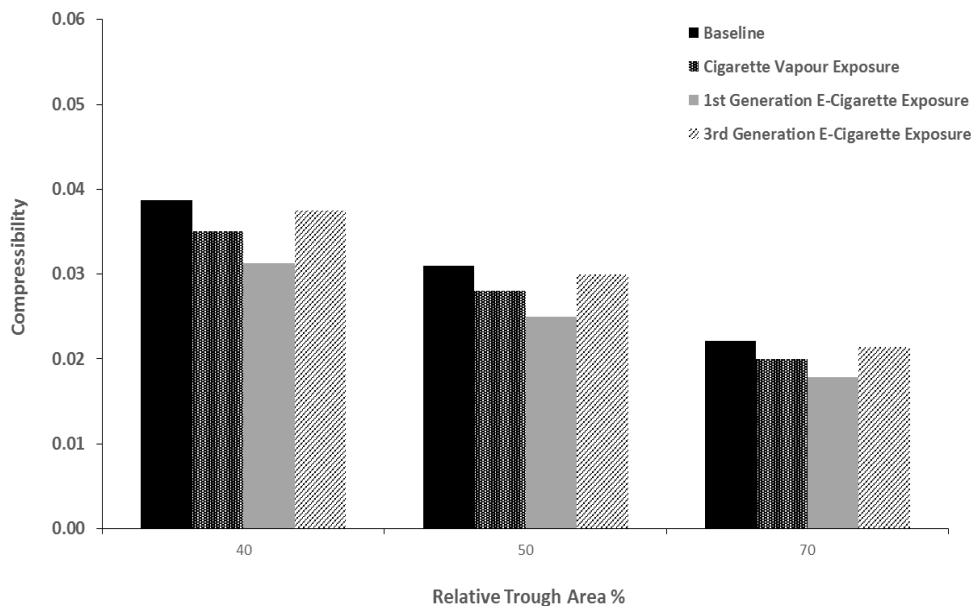
527 We attribute the apparent deviation between each Langmuir isocycle to both the loss / degradation
 528 of amphiphilic material at the air-liquid interface and the penetration of nicotine molecules between
 529 the polar head groups of the constituent molecules [11 & 33]. The reduction in the surface pressure
 530 is more pronounced upon exposure to the vapour generated from the 1st generation e-cigarette.
 531 Here, there is a clear translocation to the left within the plot when compared with baseline starting
 532 from approximately 1mN/m up towards 28mN/m. Such deviation is not as apparent shift in the case
 533 of exposure to 3rd generation e-cigarette vapour. In the case of exposure to both 1st and 3rd
 534 generation e-cigarette vapour exposure, the hysteresis between the expansion and compression
 535 phases are of similar sizes to that presented within the baseline.

536

537 3.4.1 Langmuir Isocycle Compressibility Analysis

538

539 In a similar fashion to that previously described, consideration was given to the quantitative
 540 determination of the influence cigarette / e-cigarette vapour had on simulated pulmonary surfactant
 541 compressibility during active cycling; once again Equation 1 was applied. Here, the slope of the
 542 Langmuir pressure-area isocycle was considered along the liquid-expanded to liquid-condensed
 543 transition. That is to say, between the surface pressures of 10mN/m to 30mN/m at the specific
 544 relative trough areas of 40%, 50% and 70%. Compressibility data for each system is presented in
 545 Figure 10.



546

547 **Figure 10.** The compressibility of simulated pulmonary surfactant monolayers at pre-defined relative trough
 548 areas in the absence and presence of cigarette / e-cigarette vapour. In all cases of repeated monolayer
 549 compression-expansion (i.e. Langmuir isocycles), the delivery of such vapour to the test zone decreased the
 550 compressibility term.

551 Following exposure to cigarette / e-cigarette vapour, the compressibility term decreased. Lower
552 compressibility values indicate that the surfactant film became more rigid in character and thus
553 harder to compress when compared to the baseline. This effect was more pronounced in the case of
554 the 1st generation e-cigarette vapour, demonstrating a potentially greater adverse effect on
555 pulmonary surfactant activity. As per previously noted, the influence on monolayer compressibility
556 is minimal in the case of the 3rd generation e-cigarette; this point supports the usefulness of the
557 more recently developed electronic products (e.g. PREPs) to support harm reduction within the
558 population.

559 The use of Langmuir isocycles closely represents the *in vivo* scenario. In this case, the collection of
560 amphiphilic molecules experience a two-dimensional lateral force on trough barrier movement to
561 the centre of the compartment with the phospholipid head groups less accessible to environmental
562 toxins and hence may be described as 'protected'. During surfactant compression-expansion cycles,
563 the fluid phase associated with surface active material is rapidly exchanged between the monolayer
564 interface and the adjoining surface associated reservoir [14 & 31]. As the monolayer is compressed,
565 the increase in surface pressure directs a fraction of the unsaturated lipid component (i.e. POPG)
566 away from the interfacial zone to desorb into the surface-associated, multilayer reservoir [39]. On
567 expansion, these fluid phase components stored in the surface associated reservoir support the
568 readsorption of the lipid fraction back to the interfacial zone [31]. The presence of cigarette / e-
569 cigarette vapour within the vicinity of a surfactant film inhibits such exchange mechanisms and
570 therefore alters the proportion of phospholipids within the two-dimensional monolayer [14]. As
571 such, the mechanical properties of the monolayer film are adversely affected (i.e. there is an
572 apparent increase in film rigidity) which ultimately impairs the surface tension lowering capacity of
573 the material [11].

574 This point is confirmed by the apparent decrease in monolayer compressibility and impairment in
575 the ability to reduce the surface tension term at all relative trough areas. A number of mechanisms
576 have been proposed to explain such findings and include for example the presence of oxygen
577 derived free radicals within cigarette vapour that are capable of reducing the amount of unsaturated
578 lipids (i.e. POPG) within the two-dimensional ensemble via peroxidation of double carbon-carbon
579 bonds within the acyl chains [40]. The net result is the presentation of a rigid interface that is high in
580 solid phase domains. This type of reaction involves the oxidative degradation of the amphiphilic
581 species by free radicals contained within cigarette vapour [41].

582

583

584 The oxidation of unsaturated components within a lipid monolayer (i.e. the exposed acyl chain
585 groups of the ensemble) is anticipated due to the availability of multiple double bonds accompanied
586 by methylene bridges that possess especially reactive hydrogen atoms [42]. Naturally, a reduction in
587 the liquid phase within a rigid monolayer leads to poor respread profile on expansion and reduced
588 surfactant coverage at the air-liquid interface [43].

589 The data presented within this study clearly demonstrate that exposure to cigarette / e-cigarette
590 vapour has a detrimental impact on the activity of a simulated pulmonary surfactant film. The
591 amphiphilic material forming the surfactant monolayer is central to the regulation of the surface
592 tension parameter at the alveolar air-liquid interface [14 & 21]. As such, if we take the findings
593 presented within this study and extrapolate to the *in vivo* scenario, an increase in the work of
594 breathing would be anticipated. The net effect of this would be impaired lung function, which could
595 manifest as compromised gaseous exchange within the (deep) lung, potential collapse or incomplete
596 inflation of the lung structure itself, hypoxia, oedema and quite possibly pulmonary hypertension [41
597 & 44]. Furthermore, due to such deviation from the healthy state, scope exists for longstanding
598 conditions to develop including for example chronic obstructive pulmonary disease (COPD) along
599 with interstitial lung disease. Overall, impairment to lung mechanics would be expected [44].
600 Indeed, previous work has confirmed significant reductions in phospholipid concentrations in the
601 bronchoalveolar lavage fluid obtained from those who smoke cigarettes and experience COPD [45 &
602 46]. Thus, the lung-specific adverse effects associated with cigarette smoking can reduce the quality
603 of life of the individual and increase the likelihood of premature death.

604 Over the course of recent years, e-cigarettes have become increasingly popular within developed
605 countries because of the possibility of delivering nicotine to the body in a clean format whilst
606 concurrently satisfying behavioural triggers [17, 29 & 47]. In relation to this point, during 2014 Safari
607 and co-workers documented the fact that e-cigarettes can reliably deliver nicotine to the lung whilst
608 limiting the exposure to tobacco specific toxins when compared with traditional cigarettes and the
609 use of hence it is a healthier alternative from a public health perspective [48]. However, potential
610 drawbacks to the wide spread uptake of e-cigarettes involve the lack of quality control and
611 manufacturing regulations currently in place. For instance, such regulations do not fully cover
612 aspects comprising raw material inclusion, purification stages and batch-to-batch consistency of e-
613 liquid refills; all of which can impact upon the vapour profile from the respective products [17, 18, 48
614 & 49]. Clearly, these elements require further detailed investigation.

615

616 Although not reported here, some commercially available e-liquid and cartridge refills do contain
617 chemicals that may pose potential health risks to the individual; interestingly these agents have also
618 been detected within tobacco smoke vapour [16, 17, 18, 27, 47 & 48]. For example, the cytotoxic
619 and carcinogenic substances including formaldehyde, NNN, NNK and acrolein have been identified
620 within e-cigarette vapour; all may have deleterious effects on the human body [16, 17 & 48].
621 Although the concentration of such substances is much lower than in traditional cigarette vapour,
622 alteration of pulmonary surfactant activity is possible at the alveolar air-liquid interface and this can
623 in turn initiate the presentation and development of the lung related complications / disease states
624 listed above [1, 11 & 50].

625

626 **4. Conclusion**

627

628 This study has demonstrated that exposure to cigarette / e-cigarette vapour does modify the
629 structure-function activity of simulated pulmonary surfactant monolayers under physiologically
630 relevant conditions. The results offer insight into the potential effects such (environmental) toxins
631 can have on the human lung. With reference to the dynamic system investigated herein, the
632 capacity to reduce the surface tension term was impaired throughout and the compressibility of the
633 surfactant film was reduced in all cases. The findings were ascribed to the chemical interactions
634 taking place between pulmonary surfactant-specific components and the smoke vapour delivered to
635 the test zone. We propose key mechanisms of interaction include: a) nicotine insertion into the two-
636 dimensional phospholipid ensemble, b) lipid peroxidation of the amphiphilic acyl chains and c)
637 hydrolysis of the phospholipid chains via tobacco-specific nitrosamine association.

638 Detrimental interactions such as these can cause molecular destabilisation and inhibit phospholipid
639 exchange with the surface associated reservoir system. Correspondingly, a reduction in lung
640 compliance can lead to the development of a range of lung specific complications including
641 pulmonary oedema and COPD; the latter condition is frequently noted with the chronic smoker.
642 Undoubtedly, further work is required to gain greater insight into the delicate interplay between
643 environmental toxins and the pulmonary space. Such investigation may now be readily conducted
644 via use of the lung biosimulator platform presented within this piece. Here, scope exists to consider
645 the influence of a wide range of environmental toxins have on lung function, including for example
646 petrol and diesel fumes. This device also holds potential to quantitatively probe the interaction
647 between respirable therapeutic formulations and the deep lung (e.g. in pharmaceutical dissolution
648 testing).

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650

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653

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