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1 **An Investigation into Drug Partitioning Behaviour in Simulated Pulmonary Surfactant Monolayers**
2 **with Associated Molecular Modelling**

3

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6

7 **Abstract**

8 Drug delivery to the body via the inhaled route is dependent upon patient status, device use and respirable
9 formulation characteristics. Further to inhalation, drug-containing particles interact and dissolve within
10 pulmonary fluid leading to the desired pharmacological response. Pulmonary surfactant stabilises the alveolar
11 air-liquid interface and permits optimal respiratory mechanics. This material represents the initial contacting
12 surface for all inhaled matter. On dissolution, the fate of a drug substance can include receptor activation,
13 membrane partitioning and cellular penetration. Here, we consider the partitioning behaviour of salbutamol
14 when located in proximity to a simulated pulmonary surfactant monolayer at pH 7. The administration of
15 salbutamol to the underside of the surfactant film resulted in an expanded character for the two-dimensional
16 ensemble and a decrease in the compressibility term. The rate of drug partitioning was greater when the
17 monolayer was in the expanded state (i.e. inhalation end-point), which was ascribed to more accessible areas
18 for molecular insertion. Quantum mechanics protocols, executed via Gaussian 09, indicated that constructive
19 interactions between salbutamol and integral components of the model surfactant film took the form of
20 electrostatic and hydrophobic associations. The favourable interactions are thought to promote drug insertion
21 into the monolayer structure leading to the observed expanded character. The data presented herein confirm
22 that drug partitioning into pulmonary surfactant monolayers is a likely prospect further to the inhalation of
23 respirable formulations. As such, this process holds potential to reduce drug-receptor activation and / or
24 increase the residence time of drug within the pulmonary space.

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29 *Key words*

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1. Introduction

The respiratory system can be principally divided into two regions, namely the upper and lower airways. The former marks the point of entry for atmospheric gases, respirable formulations and environmental toxins, whilst the latter is the primary site for gaseous exchange and holds the potential to be exploited for drug (i.e. insulin and analgesic) delivery to the systemic circulation [1]. Drug deposition within the respiratory tract as a whole is dependent on a number of factors including for instance inhaler technique, patient co-morbidities, device structure and function plus formulation characteristics (i.e. drug particle size, shape, density, surface energetics and external chemistries) [2]. Typically, the dose of medicine physically delivered to the lung on device activation is within the region of 20% of that emitted at source [3]. At the early stage of the drug delivery process, the aerodynamic particle size of the solid material heavily influences deposition patterns. For example, those particles of diameter 5μm or less hold a good chance of deep lung deposition with drug particles of less than 3μm diameter able to reach the alveolar space [4]. Following delivery to the deep lung and related interaction with the respective internal surfaces, individual drug-containing particles and solubilised drug molecules must overcome a number of barriers (e.g. pulmonary surfactant and the lung epithelial layer) and processes (e.g. mucociliary clearance and partitioning) prior to local or systemic activity [5]. A robust understanding of the fate of inhaled therapies, and of particular relevance to the work presented herein drug partitioning within pulmonary surfactant monolayers, can inform the drug design process and consequently lead to improved respirable formulations.

Pulmonary surfactant is central to effective respiratory mechanics. This endogenous material bathes the alveolar air-liquid interface and preserves airway patency by reducing the work of breathing [6]. In addition, the substance protects the lung from invading microorganisms, environmental toxins and particles inhaled from the atmosphere by promoting the process of mucociliary clearance [7]. The lipid element of pulmonary surfactant accounts for 90% of the blend and consists of several species such as phosphatidylcholines (PC), unsaturated phosphatidylglycerols (POPG) along with cholesterol, fatty acids and triglycerides plus palmitic acid (PA). Dipalmitoylphosphatidylcholine (DPPC) is the most abundant phospholipid within pulmonary surfactant, ranging from between 40% - 80% by weight [8]. This particular species packs tightly at the interface and reduces surface tension to near zero values from the maximum surface pressure of 70mN/m [8]. Surfactant specific proteins (SP) account for the remaining 10% of the mixture and include SP-A, SP-B, SP-C and SP-D; all differ in molecular weight, size and function [7].

73   Detailed discussion regarding the chemistries of key components of model pulmonary surfactant (i.e.

74   DPPC, POPG and PA) [9] has been provided elsewhere [10], hence consideration will be limited here.

75   In brief, the DPPC molecule exists as a zwitterion at physiological pH [11] and includes two saturated

76   acyl chains which assemble through hydrophobic interactions into gel-like condensed phases [12]. The

77   quaternary ammonium group that holds a permanent charge can act as a non-classical hydrogen bond

78   donor. In addition, a hydrogen bond acceptor is present within the molecule as a result of the

79   negatively charged phosphate group; unlike the positive charge, the negative charge is pH dependant.

80   Post compression, DPPC is unable to reform the monolayer rapidly as high surface pressures promote

81   the solid state. Therefore, additional lipid species are required to improve and facilitate material

82   respread during inspiratory phases [8]   Indeed, this particular point has been highlighted by

83   Veldhuizen and co-workers who demonstrated that DPPC:PG mixtures increased adsorption activity

84   compared to single component mixtures alone [13]. In relation to this, it is widely acknowledged that

85   surfactant specific proteins are central in promoting material respread, adsorption and stabilisation

86   of the surface film during the breathing process [14]. The 1-palmitoyl-2-oleyl-phosphatidylglycerol

87   (POPG) molecule is an unsaturated anionic phospholipid that increases the fluidity of a two-

88   dimensional surfactant film and enhances adsorption at the air-liquid interface post compression. This

89   fluidising agent has a similar chemical structure to DPPC, however the quaternary ammonium group

90   is replaced with two hydroxyl groups and bears one negative charge per molecule in the form of a

91   phosphate group [10]. Palmitic acid is composed of a 16-carbon acyl chain that makes up the fatty

92   acid component of some phospholipids and improves the surface properties of the surfactant,

93   especially DPPC. This particular molecule is a long chain saturated fatty acid with a terminal carboxylic

94   acid group. The species enhances the rigidity of a pulmonary surfactant monolayer at low surface

95   tensions and facilitates respreading; thus supporting its inclusion within model pulmonary surfactant

96   formulations considered in this work [9].

97   The application of Langmuir monolayer technology to study pulmonary surfactant relies upon the

98   careful arrangement of amphiphilic molecules across the surface of an aqueous subphase such that

99   the hydrocarbon chain components direct themselves towards the gaseous phase (i.e. air) and related

100 polar functionalities penetrate into the liquid phase (i.e. ultrapure water). Once established, scope

101 exists to apply lateral forces to the surfactant film either in isolation (i.e. Langmuir isotherm) or in

102 rapid succession (i.e. Langmuir isocycle) to probe structure-function activity.   Interestingly,

103 opportunity also presents to hold the surfactant molecules in a fixed position at a particular target

104 pressure and observe the impact of molecular interactions on material dynamics over time.

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106   Such Langmuir surface pressure – time plots, leading to penetration pressure – time data series, can

107   be readily applied to better understand the interaction between drug molecules dissolved within the

108   supporting subphase and the two-dimensional ensemble under investigation (i.e. to assist in the

109   determination of drug partitioning behaviour within a simulated pulmonary space).

110   Drug partitioning is the distribution of therapeutic molecules between two immiscible phases, where

111   an aqueous solution is usually present [15]. The ability to achieve certain concentrations in different

112   phases underpins diffusion of molecules which is fundamental in the process of drug delivery to the

113   body and in particular drug absorption leading to a therapeutic response. Clearly, the degree to which

114   drug partitioning occurs is dependent on the properties of the surrounding phases and respective

115   chemical components. Most drug molecules may be ionised in solution resulting in either anionic,

116   cationic, zwitterionic or neutral forms; the extent of which depends on the acidity or basicity of the

117   drug and relative pH of the solution. Naturally, the ionisation state of a therapeutic molecule can

118   significantly impinge upon the partition index (i.e. in terms of lipid solubility within a surfactant film

119   moving from solvent water). Additional factors that may influence drug partitioning include the size,

120   shape and concentration of the drug molecule itself [16]. To date, relatively few studies have

121   considered drug (in the present case salbutamol sulphate) penetration into simulated pulmonary

122   surfactant monolayers and rationalised the resultant biological outcomes. This fact may be ascribed

123   to the inherent complexity of the systems involved [15]. However, applied research in this field is

124   possible with evidence emerging that drug partitioning within such a space can occur via unassisted

125   thermodynamic mechanisms. For example, in 1998 Krill and colleagues highlighted that initial

126   penetration and subsequent partitioning of a drug into a two-dimensional lipid film can be either

127   enthalpically or entropically driven, or indeed both [17].

128   Salbutamol sulphate is widely prescribed within the United Kingdom for the management of asthma

129   and chronic obstructive pulmonary disease (COPD) [18]. This therapeutic agent is a short acting β_2-

130   adrenergic receptor agonist that initiates relaxation of bronchial smooth muscle post administration

131   [3]. The onset of action following inhalation is typically 5 minutes and the therapeutic effect normally

132   remains for between 3 and 5 hours [18]. The recommended daily inhaled dose of salbutamol sulphate

133   is usually 100mcg – 200mcg up to four times a day, as required [18]. The high selectivity for β_2-

134   adrenoceptors may be ascribed to the N-t-butyl group within the molecule, as detailed in Figure 1
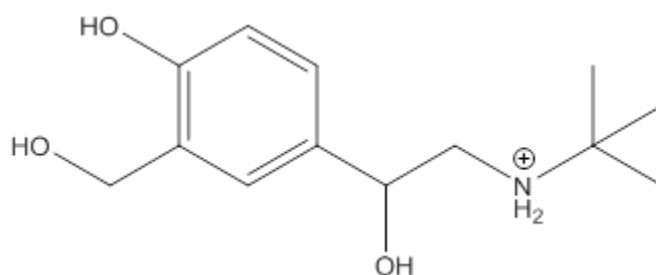
135   [19].

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137

138   **Figure 1.** *The molecular structure of salbutamol.*

139   Salbutamol presents as a racemic mixture, where the R-isomer is pharmacologically active and holds

140   high affinity for β_2-adrenoceptors as compared to the S-isomer [11]. The chemical stability of

141   salbutamol sulphate can be affected by pH, elevated temperatures and buffer solutions. As the

142   ionisation state of salbutamol varies with pH, the molecule can exist in either a zwitterionic or cationic

143   form having the two pKa values of 9.3 (i.e. amino) or 10.3 (i.e. phenolic), respectively [11, 20].

144   Protonation of the nitrogen atom within the salbutamol structure can promote ionic bond formation

145   with negatively charged functionalities of neighbouring molecules (i.e. phosphate groupings available

146   within nearby DPPC and POPG surfactant species). Furthermore, hydrogen bonds may also form with

147   the phenolic groups in salbutamol.

148   Surface electrostatic potentials relating to therapeutic drug molecules of interest (i.e. salbutamol) and

149   the polar regions of amphiphilic molecules located at the alveolar air-liquid interface (i.e. DPPC, POPG

150   and PA) may be determined via the execution of quantum mechanics protocols. Indeed, such

151   calculations have been successfully applied in recent studies conducted by Clark and co-workers

152   during 2007 [21] plus Davies and colleagues in 2017 [10]. The understanding gained can further our

153   appreciation of how interacting moieties arrange themselves when in close proximity to each other

154   and how such arrangement can dictate drug impact on system function and activity within the body.

155   Density functional theory can provide electrostatics of sufficient accuracy to explain drug-surface

156   interactions [22]. Accordingly, this approach will be applied to rationalise information obtained from

157   Langmuir monolayer studies such that deviations in isotherms / isocycles from the baseline can be

158   mechanistically explained.

159   This study aims to investigate the partitioning behaviour of our model therapeutic agent salbutamol

160   sulphate when injected to the underside of simulated pulmonary surfactant monolayers at pH 7.

161   Associated molecular modelling will be conducted to rationalise key interactions at the molecular

162   level. The results obtained will be related to the fate of drug entities on delivery to the respiratory

163   tract.

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2. Materials and Methods

2.1 Materials

Salbutamol sulphate was purchased from BUFA Chemicals, Germany (Charge: 13K26-B07-296570. Art. Nr. 13010). The surfactants DPPC (BN: 160PC-319) and POPG (BN: 160-181PG-137) were obtained from Avanti Polar Lipids, USA, whilst PA was acquired from Sigma-Aldrich, UK (BN: 087K1877). The materials were of analytical grade and used as supplied. Chloroform ($CHCl_3$) was also of analytical grade (≥ 99.9%) and purchased from Fischer Scientific, UK (BN: 1693191). This solution was employed to dissolve the surface active material to form the Langmuir trough spreading solution and for all cleaning procedures. Ultrapure water (Purite, UK), of resistivity 18.MΩcm, was used both during cleaning procedures and as the aqueous subphase during all Langmuir monolayer work.

2.2 Method

2.2.1 Langmuir Monolayers

Simulated pulmonary surfactant monolayers were generated using a Langmuir trough (Model 102M, Nima Technology, UK). Surfactant-free Kimtech tissues (Kimtech Science, Kimberley-Clark Professional, 75512, UK) were soaked in chloroform and used to clean all the glassware and contacting surfaces. Trough cleanliness was confirmed by application of surface pressure test runs, where a value of 0.4mN/m (or less) at full barrier compression confirmed suitability. A chloroform-based spreading solution composed of DPPC, POPG and PA in the ratio 69:20:11 was produced at a concentration of 1mg/ml [9]. Subsequently, a volume of 15µl of the spreading solution was applied to the surface of the aqueous subphase by drop-wise addition and a period of 10 minutes allowed to enable monolayer settling. The Langmuir trough barriers were set to move to the centre of the trough at a rate of 25cm^2/min in the case of isotherm plots. With regard to Langmuir isocycle data, the barrier system was programmed to operate at 100cm^2/min. Surface pressure vs percentage trough area readings under ambient conditions (i.e. 20°C $\pm$ 1°C) were collected using a Wilhelmy plate at the centre of the compartment.

199   To examine the rate of drug partitioning with respect to time, the target pressures of 10mN/m (i.e.

200   inhalation end-point) and 50mN/m (i.e. exhalation end-point) were established and left to stand for

201   one hour. Here, the first 5 minutes were used to condition the monolayer and salbutamol sulphate

202   was then injected underneath the monolayer at the 5th minute, as detailed in the following section.

203   Data was then acquired after the 10th minute to allow the monolayer to settle upon addition of the

204   drug. All data were acquired in triplicate and the standard error of the mean calculated accordingly.

205   The analysis of covariance was elucidated in order to test for significance within the time-based data

206   sets.

207   2.2.2   Salbutamol Sulphate Administration to Simulated Pulmonary Surfactant Monolayers

208

209   Initially, 2mg of salbutamol sulphate was accurately weighed and then dissolved in 1ml of ultrapure

210   water to obtain a stock solution of concentration 2mg/ml. This drug-containing solution was

211   subsequently diluted 5 times by removing 100µl of solution and adding this to 900µl of ultrapure

212   water. On completion of this process, the concentration of the final salbutamol sulphate solution was

213   0.02mcg/ml. Further to a period of 10 minutes for mixed surfactant monolayer spreading, the pre-

214   prepared salbutamol-containing solution was delivered to the underside of the two-dimensional film

215   (i.e. to reflect drug availability post particle dissolution). Here, a volume of 500µl of the diluted

216   salbutamol solution was added to either side of the compartment underneath the trough barriers. On

217   delivery a period of 10 minutes allowed the drug to distribute evenly within the supporting subphase

218   and interact within the simulated pulmonary surfactant monolayer. Langmuir isotherms, isocycles

219   and surface pressure – time data were then generated for each system under investigation. Each

220   investigation was repeated in triplicate with the standard error of the mean in turn calculated.

221   To investigate dose response effects, individual Langmuir isotherms and isocycles were obtained with

222   concentrations of 0.01mcg/ml, 0.02mcg/ml and 0.04mcg/ml of salbutamol; where a total of 1mg, 2mg

223   and 4mg were diluted 5 times as previously described. In this case, the dose conversion from 200mcg

224   (i.e. two standard doses) to 0.02mcg/ml was calculated as a factor of the surface area of the Langmuir

225   trough. Here, the Langmuir trough area was 70cm^2, whilst the surface area of the lung is recognised

226   to be 70m^2 [23].

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231   2.2.3 Langmuir Monolayer Analysis

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233   *2.2.3.1 Compressibility*

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235 The compressibility term relates to the ability of a surfactant film to reduce the surface tension term

236 with minimal transformation to surface area [24]. An ideal lung surfactant should have a low

237 compressibility value as this indicates the rigidness of the monolayer which represents *in vivo*

238 conditions [25]. To calculate the compressibility term, Equation 1 was employed.

239

240        $$Compressibility = \frac{1}{A} \; x \; \frac{1}{m}$$

241

242 **Equation 1.** *Calculation of the compressibility of the monolayer.*

243

244 Where A represents the relative surface area and m the slope of the isotherm. Here, 'm' was calculated

245 using 'm $= \frac{y2-y1}{x2-x1}$', between 50% and 80% of the Langmuir trough area.

246

247   *2.2.3.2 Statistical Analysis*

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249 With respect to Langmuir surface pressure - time data, statistical analysis involved application of

250 analysis of covariance using Minitab v17 [26]. This software was utilised to compare the mean of each

251 data point with time and pressure. Here, a 'p' value of <0.05 was used to demonstrate significance.

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266   2.2.4 Molecular Modelling

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269   In order to rationalise drug partitioning behaviour in proximity to simulated pulmonary surfactant

270   monolayers, system components were studied at the RHF/6-31G* level via Gaussian09 [27, 28, 29,

271   30]. Conformations of the key elements for molecular recognition at the underside of the surfactant

272   monolayer (i.e. excluding the more external hydrocarbon chain groupings) were generated using

273   omega [31]. Following geometry optimisation, the electron density was visualised in Gaussview [32].

274   Here, the electrostatic potential is projected onto a surface of constant electron density using default

275   values. Representations of the projected electrostatic potential were generated from two opposing

276   sides. The resultant output was the generation of a number of images that reflect all of the entities

277   that could potentially interact at the test interface.

278

279   **3   Results and Discussion**

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281

282   During this work we have applied a mixed surfactant monolayer composed of primary lipid species of

283   the lung (i.e. DPPC, POPG and PA) to represent the alveolar air-liquid interface within the laboratory

284   setting. Throughout, the dynamic interplay between constituents of the thin lipid films and drug

285   molecules was considered. The overarching intention was to determine the mechanism(s) of

286   salbutamol interaction with simulated pulmonary surfactant monolayers and hence better

287   understand drug partitioning behaviour further to delivery to the respiratory tract.

288

289   *3.1 Langmuir Isotherms*

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291   Langmuir pressure-area (π-A) isotherms of the mixed monolayer system following exposure to

292   increased concentrations of salbutamol sulphate are presented in Figure 2.
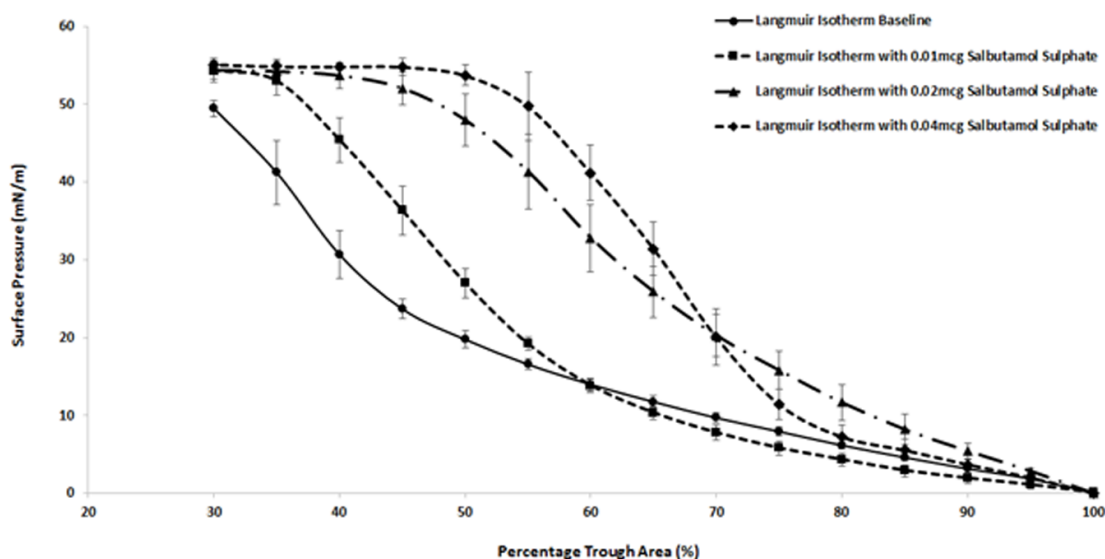
293

Figure 2. *Langmuir π-A isotherms for the mixed surfactant system supported on an ultrapure water subphase with increased concentrations of salbutamol sulphate at pH 7. In each case, a total of 3 repeats were acquired to enable the presentation of average values with error bars representing one standard error in the mean. All experiments were conducted at a temperature of $20°C \pm 1^oC$.*

On inspection of the data presented in Figure 2, it is evident that compression of the mixed monolayer system led to an increase in the surface pressure term throughout. In all cases smooth traces are apparent and are in line with previously acquired data [10]. Clear gradient changes within each curve reflect phase transitions within the two-dimensional ensemble [33]. Baseline data confirmed that the mixed surfactant film attained a maximum surface pressure of 49mN/m. However, this value increased upon salbutamol sulphate addition to the supporting aqueous subphase; the maximum surface pressure was above 54mN/m, for all concentrations of salbutamol sulphate that were studied. The apparent increase in the surface pressure term was attributed to the drug contribution at the air-liquid interface within the test zone.

Administration of salbutamol sulphate to the mixed surfactant monolayer caused a change to the Langmuir isotherm shape when compared to the baseline. Here, expansion of the two-dimensional ensemble is consistently demonstrated, being concentration dependent. The delivery of 0.01mcg salbutamol sulphate to the test zone resulted in monolayer expansion, with a maximum surface pressure of 54.3mN/m recorded; a comparable value to the 0.02mcg/ml addition. Whilst the delivery of 0.04mcg/ml salbutamol sulphate to the supporting aqueous media resulted in an increase in the surface pressure term to 55.1mN/m. This equates to a 1.5% increase in maximum surface pressure from the addition of 0.02 mcg of salbutamol. The Langmuir isotherm data also indicate that as the concentration of salbutamol sulphate increases, the curve plateau point is realised at an earlier stage.

317 For doses 0.01mcg, 0.02mcg and 0.04mcg the isotherm starts to plateau at trough areas of 35%, 40%

318 and 50% which confirms a greater solid phase contribution to monolayer dynamics.

319 On consideration of the compressibility term, a decrease in the descriptor was evident on increasing

320 salbutamol sulphate concentration as outlined in Table 1 (e.g. the addition of 0.04 mcg/ml caused an

321 approximate 70% reduction from the baseline value). The clear reduction in this parameter confirms

322 that as the number of drug molecules increase, the two-dimensional film becomes more rigid and less

323 compressible.

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Surface Area (%)	Baseline (Monolayer)	Salbutamol (0.01mcg)	Salbutamol (0.02mcg)	Salbutamol (0.04mcg)
80	0.0268	0.0163	0.0101	0.0082
70	0.0306	0.0187	0.0115	0.0093
60	0.0358	0.0218	0.0135	0.0109
50	0.0429	0.0261	0.0162	0.0130

330 **Table 1.** *Compressibility values (mN/m) of the Langmuir surface pressure isotherms*.

331 Drug insertion into a surfactant film influences lipid packing and hence system dynamics. Here, the

332 addition of salbutamol sulphate to the underside of the pulmonary surfactant monolayer, plus related

333 molecular interaction, led to increased rigidity and a more rapid increase in the surface pressure

334 term. The net effect is the presentation of the condensed / solid phases at an earlier point in time.

335 This is due to an increase in the number of molecules over a constant surface area. The process by

336 which the drug molecule can partition into the monolayer and diffuse out can also be explained by

337 the inhomogeneous solubility-diffusion mechanism [34]. This involves a three-step process by which

338 the molecule partitions in, diffuses within the structure and to an extent partitions out, which depends

339 on thermodynamic driving forces of the complete system under investigation.

340 On delivery of the drug-containing solutions (e.g. 0.02mcg/ml) to the supporting aqueous subphase,

341 a further dilution took place in the average volume of 41ml (n=3) of ultrapure water held within the

342 Langmuir trough. Upon addition of 1ml of the 0.02mcg, this would equate to a final concentration of

343 4.76×10^{-4} mcg/ml of salbutamol sulphate in the subphase. Furthermore, an addition of 0.01mcg and

344 0.04mcg would further dilute the drug to a concentration to 2.38×10^{-4} mcg/ml and 9.52×10^{-4} mcg/ml,

345 respectively.

346   This indicates that with highly diluted drug concentrations scope exists for partitioning into the

347   monolayer, which is shown by translocation of the curves to the right. Indeed, this effect was reported

348   by Jablonowska and Bilwicz during 2007 where the group demonstrated that ibuprofen diluted to

349   concentrations of $2x10^{-5}$- $2x10^{-4}$ M caused changes in the compressibility of a surfactant monolayer

350   with partitioning still occurring at low concentrations [35].

351

352   *3.2 Langmuir Isocycles*
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355   Average Langmuir compression-expansion cycles of the simulated pulmonary surfactant monolayer

356   system at pH 7 pre- and post-salbutamol sulphate addition are presented in Figure 3. The data

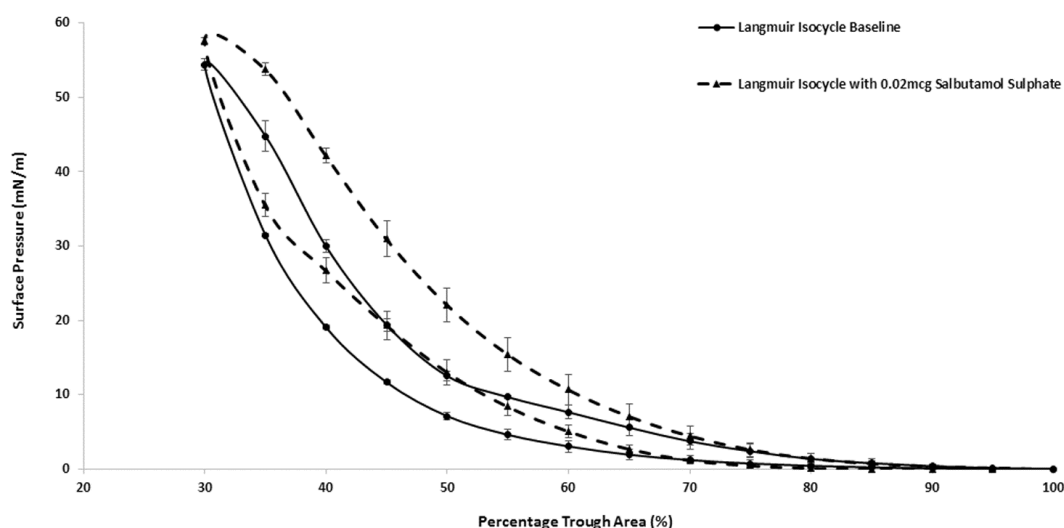357   presented are averages of three replicates of the same.

358

359   **Figure 3.** *Average Langmuir π-A isocycles for the mixed surfactant system supported on an ultrapure water*
360   *subphase with a salbutamol sulphate concentration of $4.76x10^{-4}$ mcg/ml at pH 7. In each case, 3 repeats were*
361   *acquired and points are the mean with error bars of one standard error in the mean. The experiments were*
362   *conducted at a temperature of 20°C $\pm$ 1°C.*

363

364   During Langmuir pressure – area isocycle generation, initial pre-conditioning inward and outward

365   sweeps were executed (n=4). The purpose of this procedure was to prepare the conformation of the

366   mixed monolayer system to best represent that noted within the (deep) human lung. Clearly, this

367   approach differs from a single compression isotherm in that the constituent molecules are arranged

368   more favourably and typical of the physiologically relevant scenario.

369   This pre-conditioning stage and removal of associated early phase traces (n=4) results in the

370   elimination of the solid phase plateau noted in single Langmuir pressure – area isotherms (i.e. those

371   shown in Figure 2).

372   On monolayer cycling, the maximum surface pressure for the mixed surfactant system was 54.4mN/m.

373   However, on addition of salbutamol sulphate this value increased to 57.6mN/m; equating to a 6%

374   increase in the term. Once again, the data indicate that further to the addition of salbutamol sulphate

375   to the supporting aqueous media the monolayer becomes expanded in nature (i.e. the Langmuir π-A

376   isocycles translocate to the right). In a similar fashion to that outlined above, the compressibility term

377   also decreased following salbutamol sulphate administration.   Here, the baseline value of

378   0.0288mN/m decreased to 0.0203mN/m, representing a 30% reduction in the parameter indicative of

379   reduced flexibility, compressibility and increased rigidity of the monolayer.

380   On completion of the Langmuir isocycle experiments, deviation in the gradient of the trace confirmed

381   amphiphilic molecule phase transitions (i.e. movement from the gaseous phase through to the

382   expanded and condensed phases and ultimately the solid phase). On moving from the gaseous phase

383   to the solid phase, there is a related increase in molecular order. As such, at low surface pressures

384   the monolayer exhibits a certain level of disorder with some spacing between constituent surface

385   active molecules. At the higher surface pressure, the monolayer is decidedly ordered and a tighter

386   molecular packing of DPPC, POPG and PA has occurred resulting in a solid phase transition [36]. Thus,

387   the very state of the surfactant film will govern the propensity of the dissolved drug molecules to

388   partition into the ensemble.

389   The injection of salbutamol sulphate (0.02mcg/ml) into the supporting aqueous subphase caused the

390   gradient of the Langmuir isocycle to become steeper. The data indicate that the phase transitions

391   occur at an earlier point in time, as compared to the baseline. The result can be attributed to an

392   increase in the number of molecules across the two-dimensional plane (i.e. the insertion of drug

393   molecules into the surfactant monolayer). It is the very presence of drug molecules within the system

394   that leads to a more rapid phase transformation and resultant tighter packing at an earlier stage. Such

395   packing causes the presentation of the solid phase sooner than compared to the baseline.

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399 Monolayer collapse occurs where lipid components become unstable after maximum surface pressure

400 has been achieved [37]; naturally this situation should be avoided within the laboratory setting. In

401 terms of the Langmuir isotherm baseline data, the simulated pulmonary surfactant monolayer

402 collapsed at the maximum surface pressure of 49.5mN/m. However, typical collapse pressure for a

403 simulated pulmonary surfactant monolayer (i.e. Curosurf® [38]) would be approximately 70mN/m.

404 This parameter is influenced by factors such as the operating temperature and the lipid composition.

405 Thus, we suggest that the lower maximum surface pressure present herein was a combined function

406 of the operating temperature (i.e. 21°C), monolayer composition (i.e. only DPPC, POPG and PA), plus

407 monolayer pre-conditioning stages that involved four repeated compression – expansion events.

408 It is anticipated that to some extent drug partitioning within a surfactant film is reversible as re-

409 spreading occurs on barrier expansion (i.e. representative of inhalation) [37]; particularly at the higher

410 percentage trough areas. Excess lipid material is removed from the subphase into the surface

411 associated reservoir during compression at exhalation. The material is usually composed of

412 unsaturated lipid components where this phenomenon illustrates the molecules are being 'squeezed

413 out' from the monolayer. As a result, saturated lipid components at the interface obtain low surface

414 tensions *in vivo.*

415 *3.3 Langmuir Surface Pressure – Time Analysis*

416

417 During this work, consideration was given to how the physical state of a simulated pulmonary

418 surfactant monolayer (i.e. expanded or compressed) can influence drug partitioning behaviour. To

419 this end, Langmuir surface pressure – time plots were generated. The penetration pressure ($\Delta\pi$) [17,

420 39] data for both the expanded and compressed systems are presented in Figure 4.
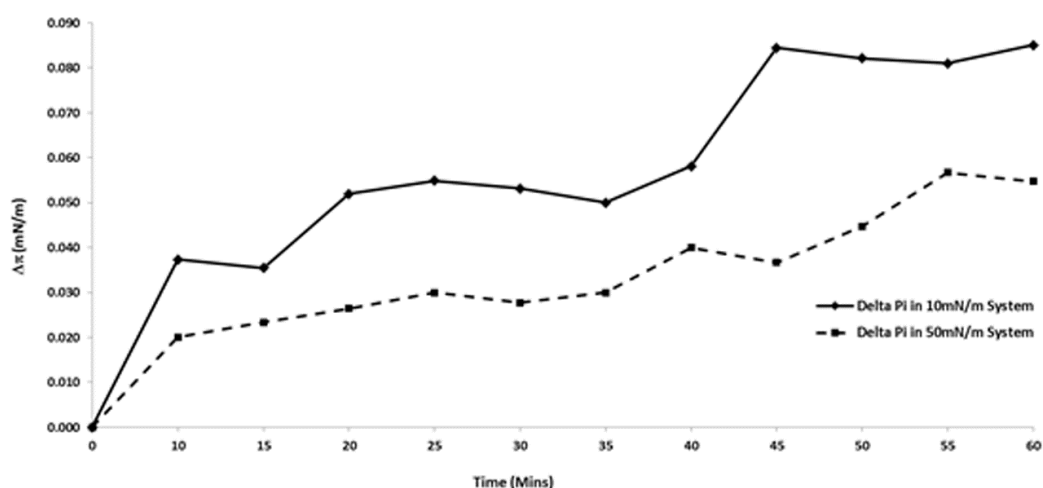
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Figure 4. *Average Langmuir penetration pressure – time plots for the systems under consideration. Each data point arises from three repeats of the same experiment. Standard error of the mean bars have been included within the plot, however visibility is limited due to low variability in the data sets. The surface pressure term has an impact on the partitioning behaviour of salbutamol, with the lower surface pressure offering greater scope for drug insertion into the two-dimensional film. All data are acquired at pH 7 and a temperature of 20°C $\pm$ 1°C.*

427

The addition of salbutamol sulphate (0.02mcg) to the underside of the simulated pulmonary surfactant monolayer caused an increase in the surface pressure term in both cases with respect to time. Upon inspection of the data presented in Figure 4, it is evident that the change in surface pressure ($\Delta\pi$) is greater in the case of the more expanded system (i.e. 10mN/m) and this directly aligns with the relaxed physical arrangement of the surfactant molecules. Thus, there is a greater propensity for drug molecules to partition into the two-dimensional surfactant film at the lower surface pressure (i.e. inhalation end-point). We emphasise that at the target pressure of 50mN/m, the rate of drug partitioning was slower albeit still taking place (i.e. P=0.0014: Langmuir surface pressure – time data, not shown). Whilst there would be tight molecular order at this pressure, absolute compression does not occur and consequently there is still the opportunity for salbutamol to interact constructively with and partition into the monolayer.

442   The data confirm that the physical state of the monolayer can have a significant bearing on the

443   partitioning behaviour of solubilised drug molecules within the underlying vicinity. Typically, the polar

444   head groups of DPPC, POPG and PA are situated deep within the supporting aqueous media at low

445   surface pressures [40, 41]. Thus, a solubilised drug molecule (i.e. arising post drug particle dissolution)

446   can readily interact with the components of the surfactant monolayer and subsequently associate

447   with or penetrate into the two-dimensional structure. It is to be expected that the extent of drug

448   partitioning is likely to be greater at a lower surface pressure (i.e. the point of inhalation) due to the

449   increased likelihood of accessible regions [42, 43] for molecular insertion.

450   Indeed, when we consider the two-dimensional arrangement of the components of a model lung

451   surfactant (i.e. DPPC, POPG and PA) throughout the course of compression and expansion, there is

452   high level of certainty that 'accessible regions' will present to in turn promote drug partitioning. At

453   this juncture, it is appropriate to refer to the work conducted by Bringezu and colleagues in 2003 who

454   probed the impact of environmental tobacco smoke on the primary lipid species of lung surfactant

455   [9]. During the work, the group applied fluorescence microscopy to observe the changes in monolayer

456   structure as lateral compression was applied across the plane. With regard to their pristine system

457   (i.e. identical to that applied during this work), low surface pressures were linked to the presentation

458   of condensed or tightly packed DPPC / PA regions within expanded or more relaxed DPPC / POPG areas

459   consistently visible throughout the surfactant film as a whole. The fluorescent dye applied during the

460   work preferentially distributed itself into the disordered DPPC / POPG regions. As the surface pressure

461   was ramped towards the collapse point, the number of solid phase domains increased. Thus, at the

462   higher surface pressures the monolayer structure became much more ordered. The data presented

463   within the piece confirmed that a surfactant monolayer composed of primary lipid species exhibits

464   non-uniform packing throughout, a feature to be anticipated at the alveolar air-liquid interface within

465   the body.

466   The apparent lack of homogeneity across a lung surfactant film does lend strong support to the

467   concept of 'accessible areas' to promote drug partitioning, as detailed by Vilallonga and Phillips in

468   1978 [39]. This work considered how the anthracycline glycoside antibiotic doxorubicin associated

469   with phospholipid monolayers located at the air-liquid interface. On application of the accessible area

470   calculation (i.e. $a = A - NA_m$) it was established that the condensed monolayer had an average 7%

471   region of access for dissolved drug molecules, as compared to the less condensed monolayer of 33%

472   availability for the same.

473

474   The net effect was a greater increase in the surface pressure term for the more relaxed monolayer
475   system (i.e. there was more area accessible for drug partitioning and as such more drug molecules
476   were able to penetrate into the structure and increase the surface pressure). The group also
477   demonstrated an increase in the surface pressure term in all cases following the injection of drug
478   substance beneath the monolayer structure. The result was ascribed to the 'osmotic approach'
479   leading to an increase in the number of molecular entities at the interface.

480   A similar principle was applied by Krill and co-workers in 1998 who considered the partitioning
481   behaviour of various β-antagonists (e.g. propranolol, oxprenolol, metoprolol and nadolol) when
482   placed in an aqueous environment beneath Langmuir monolayers composed of
483   dimyristoylphosphatidylcholine [17]. Within the study, clear reference was made to the fact that all
484   molecules demonstrated surface activity, irrespective of solution concentration.   Importantly,
485   movement into condensed phases of the monolayer structure was noted, which links with the findings
486   presented herein (i.e. a notable change in the presented penetration pressure at the higher value of
487   50mN/m). Thus, throughout tidal breathing one would expect therapeutic entities to associate with
488   and partition into lung surfactant at the alveolar air-liquid interface, irrespective of the surface
489   pressure placed on the endogenous material at any one time. The propensity of this process heavily
490   depends upon the chemical properties of the administered molecule(s). For instance, Krill and
491   colleagues demonstrated that propranolol exhibited the greatest degree of monolayer penetration
492   followed by metoprolol, oxprenolol and finally nadolol. Thermodynamic aspects are central to drug
493   insertion into a surfactant film. For example, Krill noted that propranolol partitioning into the
494   monolayer structure was enthalpically and entropically driven, and this contrasted sternly with
495   nadolol which was mainly enthalpically driven whilst being strongly entropically hindered. It would
496   appear that two key aspects dominate the interaction as a whole; namely, modification to the
497   monolayer structure across the plane plus the physical movement of drug molecules into the lipid
498   layer.

499   Variation in the monomolecular structure results further to drug partitioning with either the drug
500   causing expanded regions to become more condensed in nature via insertion within accessible areas
501   or direct interaction between drug molecule and surfactant components, as modelled herein. Such
502   modification to the surfactant film in the (deep) lung can influence structure-function activity [10],
503   however in general terms the surfactant film appears robust and resilient to external stressors (i.e.
504   drug substances and environmental toxins [43]) and this allows it to fulfil its crucial biological function.
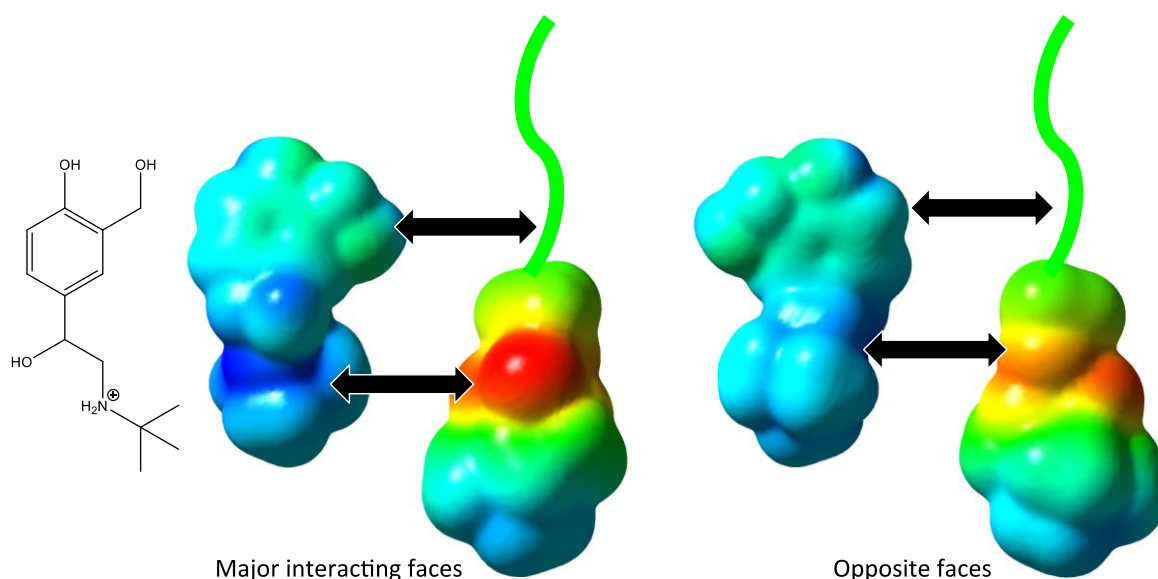
505

506

507   *3.4 Molecular Modelling*

508

509   *3.4.1 Salbutamol Interaction with Surfactant Film Components*

510

511 The electrostatic potential surfaces (EPSs) of the polar head groups associated with each surfactant

512 molecule (i.e. DPPC, POPG and PA) along with salbutamol were calculated via the quantum mechanics

513 software package Gaussian09. The hydrophobic tails were abbreviated to a methyl group, to avoid

514 studying conformations that would not be relevant to the monolayer conditions. A set of up to 10

515 conformations of each molecule was created by omega and each was optimized with RHF/6-31G*.

516 The lowest energy conformation was then identified and its electrostatic potential projected onto a

517 surface of the molecule (the total electron density cut at 0.0004 electrons/Å^3). In these electrostatic

518 potential maps, red indicates strongly negative regions, yellow less negative regions, blue strongly

519 positive regions and cyan less positive regions. Regions coloured green have an approximately neutral

520 electrostatic potential. To aid analysis we provide arrows to highlight important regions of interaction

521 and place the linkage to the hydrophobic tails at the top of each figure.

522 In the case of salbutamol, one end of the molecule is generally hydrophobic (the aromatic ring) and

523 this is assumed to prefer contact with the hydrophobic tails of the monolayer and so is also placed at

524 the top of the figure. The EPSs for salbutamol are shown for the molecule in its predominant cationic

525 form. Similar images for the zwitterionic form, in which the phenol is deprotonated, were also

526 generated but represent only a small contribution (i.e. <1%) to the population of molecules and so are

527 not shown here.

528

529

Major interacting faces   Opposite faces

530

531   **Figure 5.** *EPS calculations of salbutamol and DPPC (front and rear views provided) with key interaction sites*
532   *determined for pH 7.*

533   When in proximity to DPPC, the predominant component of the monolayer, the two are likely to
534   interact in a way that maximises both electrostatic and hydrophobic interactions. As shown in Figure
535   5, when the major interacting face of salbutamol presents a large positively charged patch in such a
536   way that it can interact with the negatively charged patch to the major interacting face of DPPC, this
537   naturally places the hydrophobic aromatic ring in proximity with the hydrophobic tails of DPPC. When
538   the molecules are paired like this, it can be seen that the opposite face of each molecule are also
539   complementary, albeit with less extreme electrostatic components. When the interactions with the
540   other components of the monolayer are considered, as shown in Figure 6, it is clear that there are a
541   range of positions that salbutamol can adopt to allow it to maximise beneficial interactions with the
542   molecules around it, regardless of which molecules those are. The optimum position in each case
543   involves a different degree of penetration into the monolayer.

544   With POPG, the interactions are likely to be best when the salbutamol penetrates deeply into the
545   monolayer whereas with PA, the opposite is the case. This differing behaviour will cause different
546   effects on the monolayer. When amongst the head groups (as with PA), the salbutamol is promoting
547   the movement apart of the hydrophobic tails and making the monolayer more like the gas phase.
548   Whereas, when the salbutamol penetrates amongst the tail groups (as with POPG and DPPC), it
549   compresses those groups making the system more like the solid phase.
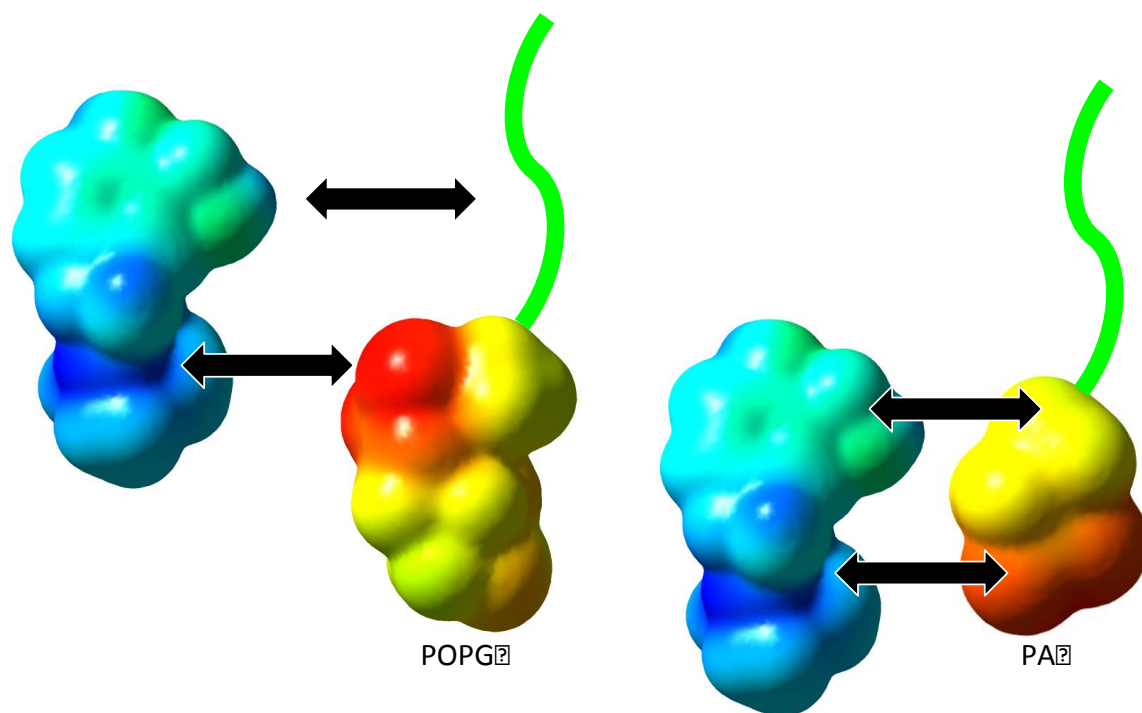
550

Figure 6. *EPS calculations of salbutamol, POPG and PA with key interaction sites determined for pH 7.*

These hydrophobic and electrostatic forces of attraction explain the consistent shift of the Langmuir isotherm and isocycle data as compared to the baseline, which relates to expansion of the monolayer. The spontaneous movement of drug molecules into the monolayer and the formation of molecular interactions between the molecules encourage and facilitate drug partitioning and insertion. Thus, the monolayer is less compressible and highly rigid resulting in an expanded character.

4. Conclusion

This study has considered the partitioning behaviour of salbutamol (sulphate) when in close proximity to fundamental components of endogenous pulmonary surfactant. The work confirms the suitability of Langmuir monolayers to serve as model interfaces to further current understanding within this relatively under-researched field. The data indicate that the drug molecule of interest impacted upon the activity of simulated pulmonary surfactant during compression and expansion phases; reflective of the human breathing cycle. The injection of salbutamol sulphate to the underside of the lipid film (i.e. to reflect drug availability post particle dissolution) caused general expansion of the surface active material and decreased the compressibility term. Drug partitioning behaviour was highly dependent on the physical state of the surfactant monolayer, where the rate of partitioning was greater at the lower surface pressure that represented greater molecular disorder.

571 Naturally, potential exists to exploit the drug partitioning process in lung surfactant films for
572 therapeutic advantage. The sustained presence of a drug molecule(s) in the pulmonary space could
573 be of clear benefit of the patient in reducing the frequency of daily dosing. Indeed, this approach may
574 be viewed as an advanced modified release strategy (i.e. non-classical) that is solely dependent on
575 chemical complementarity between all species involved.

576

577   5. **Acknowledgements**

580

581   **6. References**

582

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