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Development of a dry powder insufflation device with application in *in vitro* cell-based assays in the context of respiratory delivery

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ABSTRACT

Research on pharmaceutical dry powders has been increasing worldwide, along with increased therapeutic strategies for an application through the pulmonary or the nasal routes. *In vitro* methodologies and tests that mimic the respiratory environment and the process of inhalation itself are, thus, essential. The literature frequently reports cell-based *in vitro* assays that involve testing the dry powders in suspension. This experimental setting is not adequate, as both the lung and the nasal cavity are devoid of abundant liquid. However, devices that permit powder insufflation over cells in culture are either scarce or technically complex and expensive, which is not feasible in early stages of research. In this context, this work proposes the development of a device that allows the delivery of dry powders onto cell surfaces, thus simulating inhalation more appropriately. Subsequently, a quartz crystal microbalance (QCM) was used to establish a technique enabling the determination of dry powder deposition profiles. Additionally, the determination of the viability of respiratory cells (A549) after the insufflation of a dry powder using the developed device was performed. In all, a prototype for dry powder insufflation was designed and developed, using 3D printing methods for its production. It allowed the homogenous dispersion of the insufflated powders over a petri dish and a QCM crystal, and a more detailed study on how dry powders disperse over the supports. The device, already protected by a patent, still requires further improvement, especially regarding the method for powder weighing and the efficiency of the insufflation process, which is being addressed. The impact of insufflation of air and of locust bean gum (LBG)-based microparticles revealed absence of cytotoxic effect, as cell viability roughly above 70 % was always determined.

1. Introduction

Respiratory drug delivery is a continuously growing field of which aerosols are a relevant cornerstone. Encompassing both pulmonary and nasal delivery, the produced aerosols must comply with specific aerodynamic requirements which are different for each of the routes and even in different target areas of the lung (A.H. de Boer et al., 2022; A.H. de Boer et al., 2022). Lung inhalation may either involve liquid formulations administered by Metered Dose Inhalers (MDIs) or nebulisers, or the solid counterparts in the form of dry powders. The latter are currently the most relevant of pulmonary formulations (Zhong et al., 2018), which have higher market expression and are in evident progression not only because of the higher stability, but also due to sustainability issues. Dry powders are also a growing market in nasal delivery (Elmowafy and Soliman, 2023). The appearance of particle engineering techniques has been beneficial to improve the properties of the developed dry powders for inhalation. Their performance simultaneously depends on the biological and physicochemical aspects of the anatomical regions under consideration (respiratory tree and nasal cavity) (Mekonnen et al., 2022; Nahar et al., 2013). Formulation development and the respective preclinical studies involve *in vitro* and *in vivo* tests focused on the assessment of biological performance. *In vitro* tests intend to recreate, as far as possible, the *in vivo* setting and, thus, validate the obtained results without the need to use animal models or, at least, provide initial indication on the behaviours. Adequate *in vitro* platforms are, therefore, crucial to obtain good correlations and to

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Table 1
Spray-drying parameters used to produce the representative dry powder formulations for the insufflation assays.

Name	Concentration	T_{inlet} (°C)	T_{outlet} (°C)	Aspirator (%)	Feed flow (mL/min)	Reference
LBG	1 % (m/v)	130	84 ± 2.4	100	0.90 ± 0.01	See text
KGM/Leu	1.5/0.75 % (m/v)	170	104 ± 2.0	85	0.83 ± 0.01	(Guerreiro, 2015)
DS	2 % (m/v)	115	54 ± 1.4	65	2.7 ± 0.1	(Musacchio and Grenha, 2017)

DS: dextran sulfate; KGM konjac glucomannan; LBG: locust bean gum; Leu: l-leucine.

comply with the international guidelines that foster the reduction of animal experimentation (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, 2010). Nevertheless, mimicking the process of aerosolization process *in vitro*, as well as recreating the lung environment in the specific branch of lung delivery is not an easy task, which may compromise drawing conclusions. Initial stages of biological assessment of drug formulations typically involve cell-based assays. When the inhalable formulation comprises a dry powder, it should be tested as such. However, most frequently dry powders are tested in suspension, including in toxicological and permeability assays (Fernández-Paz et al., 2021; Thiyagarajan et al., 2021), essentially due to the absence of simple devices permitting powder aerosolization over cell cultures. Typically, the powder is mixed with the cell culture medium and subsequently incubated with the cells. Regrettably, this immersion strategy does not meet the characteristics of respiratory routes, as neither the lung nor the nasal cavity display abundant liquid (Fröhlich et al., 2016). *In vitro* platforms enabling the assessment of dry powders in cell-based studies are scarce. The existing ones are expensive and technically complex, and not feasible for early stages of research (Lenz et al., 2009; Metz et al., 2018) requiring easier solutions to address, for example, cytotoxicity or drug permeation assessment. As referred, one of the great limitations comprises the proper aerosolization of the powder over the cells, as specific devices for the process are not easily accessible to the scientific community working in the field. The purpose of this work was to develop a prototype for the delivery of dry powders onto cell surfaces, aiming at simulating more appropriately the process of inhalation. Quartz crystal microbalance (QCM) was used for dosimetry tests and also to determine the deposition profile of tested dry powders, which allows investigating their aerodynamic properties. Three microparticle formulations were used as model dry powders and a final proof-of-concept was performed using the developed prototype to determine the viability of respiratory cells after aerosolization of dry powders.

2. Materials and methods

2.1. Materials

Locust bean gum (LBG), d-mannitol, isoniazid, l-glutamine solution (200 mM), non-essential amino acids solution, penicillin/streptomycin (10,000 units/mL, 10,000 g/mL), trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA), trypan blue solution (0.4 %), thiazolyl blue tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), dimethylformamide (DMF), Triton-X 100, phosphate buffered saline (PBS) tablets pH 7.4, dimethyl sulfoxide (DMSO) were acquired from Sigma-Aldrich® (Germany). Fetal bovine serum (FBS) was purchased from Gibco (Life Technologies, USA), Dulbecco's modified Eagle's medium (DMEM) from BioConcept (Switzerland) and RPMI 1640 from Lonza Group AG (Switzerland). Konjac glucomannan and RFB were acquired from CHEMOS (Germany), dextran sulfate from Alfa Aesar (Germany) and l-Leucine from Panreac AppliChem (Germany). Ultrapure water (Milli-Q, Millipore, Watford, UK) was used throughout. All other chemicals were reagent grade.

2.2. Preparation of dry powders

Polysaccharide-based dry powders were produced by spray-drying (Buchi B-290 laboratory mini spray-dryer, Buchi Labortechnik AG, Switzerland) with varying atomization parameters that were dependent on the specific samples. Although thirteen dry powders were used to perform initial stages of the experimental work described in this manuscript, three were selected as the most representative, as described in Section 3. Their composition and spray-drying parameters are shown below (Table 1).

For the preparation of LBG microparticles, a dispersion of the polymer was prepared in ultrapure water (1 %, m/v), under stirring at 85 °C for 1 h for solubility enhancement. After cooling to room temperature, the dispersion was left under mild stirring overnight. The next day, the LBG dispersion was heated again (85 °C) before spray-drying, to decrease its viscosity. The apparatus was equipped with a high-performance cyclone and the conditions set as: spray flow rate of 473 L/h, inlet temperature of 130 °C, aspirator at 100 % and feed flow rate at 1.0 mL/min.

2.3. Characterisation of dry powders

The morphology of the microparticles composing the dry powders was determined by field emission scanning electron microscopy (FESEM; FESEM Ultra Plus, Zeiss, Oberkochen, Germany), using a previously detailed method (Guerreiro et al., 2021). The size of the microparticles was determined as the Feret's diameter by the manual measurement of 300 particles, registered from the photographs obtained by FESEM.

A theoretical aerodynamic diameter was calculated whenever possible using Eq. (1).

$$d_{aer} = d_F \sqrt{\frac{\rho_{particle}}{\rho_0 \chi}} \quad (1)$$

where d_F corresponds to the geometric size of the particles, $\rho_{particle}$ refers to the density of the particles, ρ_0 specifies the unit density (1 g/cm^3) and χ is the shape factor, that varies between 1 (spherical) and 2 (irregular) (Gupta et al., 2014).

2.4. Design of the device and printing process

The device was idealised and designed using a CAD (computer aided design) software. It was exported as a .stl file to a commercial 3D printer, and printed using stereolithography (Huang et al., 2020). The design of the device and printing of the prototype considered two pieces, one comprising a small weighing head, designed to weigh the dry powder, and another as the main body, conceived to allow powder dragging and deposition over cell layers (see Figs. 1 and 2, below).

2.5. Insufflation optimisation

Five accessories used to blow air were tested: lens blower, bicycle pump, syringe, garden spraying device and an air compressor. The capacity of these pumps to generate an air stream capable of dispersing dry powders was evaluated. In subsequent tests, the capabilities of the air compressor were further investigated, concerning the different outlet air

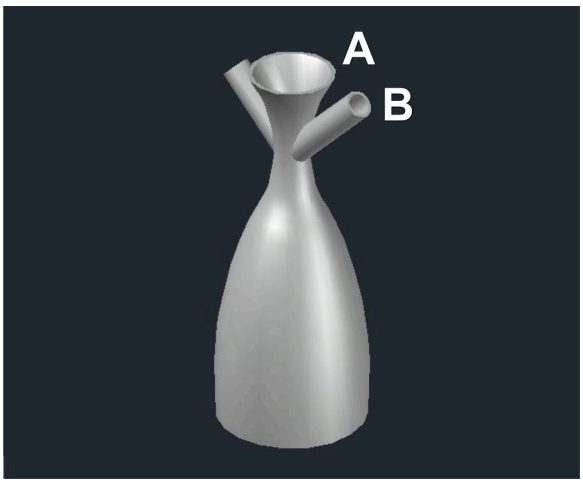


Fig. 1. Design of the body of the prototype, consisting of a single piece. The entry point for the air is one of the arms (B). The powder weighing head will be placed on top (A).

Fig. 2. Design of the powder weighing head. Powder is to be weighed in the platform. The curves in the margins maintain the adapter free of 90° angles, not promoting powder trapping upon insufflation.

pressures (30–60 psi). For the insufflation tests, the used air pistol required 30 quick activations, so that the air blown into the aerosolization device would drag the dry powder in quantifiable amounts. For the QCM and proof-of-concept tests, due to the sensitivity of this sensor, the insufflation method was optimised, in which the number of activations of the air pistol was reduced from 30 to 10 at an outlet air pressure of 30 psi.

2.6. Quartz crystal microbalance (QCM) experiments

Maxtek quartz crystals (5 MHz, Inficon) were used, which were previously coated with optically flat polished gold electrodes on both sides, and cleaned before use with absolute ethanol, ultrapure water and a piranha solution, the latter being comprised of 3:1 mixture of sulphuric acid and 30 % hydrogen peroxide and applied for 15 min. Cleaned crystals were mounted on a kynar crystal holder (Maxtek CHT-100), which was connected to a universal frequency counter (Agilent 53131A) through a phase-locked oscillator (Maxtek PLO-10i). The frequency counter was connected by a General Purpose Interface Bus (GPIB) to a computer running a data acquisition and control program developed in Keysight Visual Engineering Environment (VEE, Pro-version 9.33, Agilent) for the real time acquisition of the QCM frequency.

2.7. Insufflation assays

Thirteen polysaccharide-based microparticles developed for lung applications, previously prepared by spray-drying as described above, were chosen to test the prototype (Musacchio and Grenha, 2017; Rodrigues et al., 2017; Rodrigues et al., 2020; Guerreiro et al., 2019). Before the tests, all parts of the prototype were weighed. Afterwards, 5 – 10 mg of dry powder were weighed in the weighing head, the prototype assembled and placed on the QCM, more specifically on top of the crystal holder (Maxtek CHT-100), where a clean crystal was previously mounted. The thirteen dry powders were used to optimise the insufflation method from 30 to 10 quick activations from an air compressor (Goldair; outlet pressure set at 30 psi) to ensure that the mass of air would result in the dispersion of the dry powder. After conclusion of the test using the prototype, its parts were weighed, the frequency data saved, and images of the crystal and the prototype recorded after powder deposition.

2.8. Aerosol characterisation: deposition profile and yield of insufflation

2.8.1. Dry powder deposition profile

In the QCM, the variation of the frequency was recorded as function of time between 0 and 300 s. The data generated allowed the drawing of a graph detailing the frequency change in different events along the assay: a first equilibrium, which establishes the baseline, a frequency change when air and dry powder contact with the crystal and finally, a second equilibrium, in which a new frequency is determined due to mass change.

2.8.2. Yield of insufflation

The amount of insufflated dry powder deposited on the surface of the crystal mounted on the crystal holder of the QCM was obtained, according with the simplified Sauerbrey equation (Sauerbrey, 1959) (Eq. (2)):

$$\Delta f = -C_f \times \Delta m \tag{2}$$

in which, Δf represents the difference in frequency between the states with and without powder at a given point, C_f is the sensitivity factor of the crystal, in this case, 0.056 $Hz/ng/cm^2$ for a 5 MHz crystal at 20 °C, and Δm is the change in mass per unit area (g/cm^2). Afterwards, a yield of insufflation was determined using Eq. (3),

$$Yield\ of\ insufflation\ (\%) = \frac{Amount\ of\ powder\ deposited}{Amount\ of\ powder\ weighed} \times\ 100 \tag{3}$$

in which, the initial amount of powder weighed and inserted in the device corresponds to *Amount of powder weighed* and the amount of powder that is insufflated, reaching the base of the device corresponds to *Amount of powder deposited*.

A relative measure between powders was established as the degree of insufflation, where the highest yield obtained, recorded for the dry powder of LBG, was assumed as 100 %. The yields obtained for the remaining dry powders were calculated by adjusting to this reference (Eq. (4)),

$$Degree\ of\ insufflation = \frac{Average\ yield\ of\ insufflation\ of\ powder}{Average\ yield\ of\ insufflation\ of\ LBG} \tag{4}$$

where *average yield of insufflation of powder* pertains to the average yield result of a specific dry powder being tested, while on the denominator *average yield of insufflation of LBG* pertains to the average yield result of LBG, as it was the highest obtained in the experiments.

2.9. Cell culture

Human lung epithelial adenocarcinoma cells (A549) were provided by the American Type Culture Collection (ATCC, USA) and used in passages 24–30. Cells were cultured in flasks (75 cm^2), at 37 °C, in an incubator with 5 % CO_2/95 % humidified atmospheric air. Cell culture medium (CCM) was DMEM, supplemented with l-glutamine 200 mM (1

%, v/v), non-essential amino acids (1 %, v/v), penicillin/streptomycin at 1 % (v/v) and FBS at 10 % (v/v). CCM was exchanged two to three times weekly.

2.10. Proof-of-concept

The proof-of-concept of the developed device was performed in an assay that used the device to determine the viability of A549 cells after exposure to a dry powder composed of microparticles of LBG, selected as testing sample. Cells were seeded in petri dishes ($\varnothing = 35$ mm, 9.4 cm^2) at 3×10^5 cells/plate and incubated overnight. CCM was removed on the day after and dry powders were insufflated onto the cultured cells at a concentration of 85.8 μg/cm^2. Afterwards, 500 μL of CCM without phenol red were added to the petri dishes to avoid desiccation of the cells and exposure times of 3 h and 24 h were tested. CCM only and the insufflation of air (without microparticles) were used as controls, as well as a 2 % SDS solution (w/v). After the defined exposure time, CCM and samples were removed, and replaced by 750 μL of a solution of MTT (5 mg/mL in PBS pH 7.4), which remained under incubation for 2 h. After that, 1.5 mL of DMSO were added to each plate to solubilise the formed formazan crystals. The absorbance was read by spectrophotometry (Infinite M200; Tecan, Austria) at 540 nm, with background correction at 640 nm. The cell viability was calculated using Eq. (5).

$$Cell\ viability\ (\%) = \left[\frac{A - S}{CM - S}\right] \times 100 \quad (5)$$

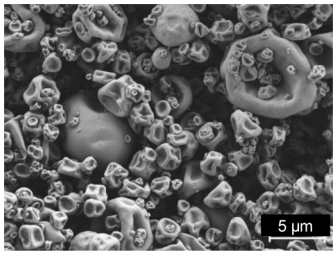
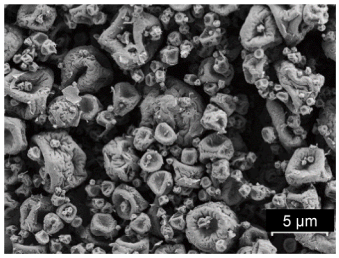
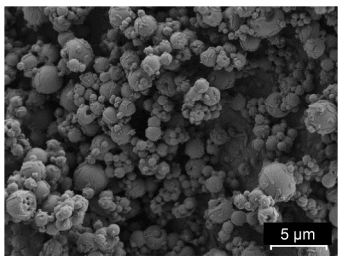
where A is the absorbance obtained upon exposure to each example, S represents the absorbance measured for SDS 2 % and CM is the absorbance read for the cells incubated in CCM. The assay was performed at least three times, with three replicates for each tested concentration.

2.11. Statistical evaluation

2.11.1. For insufflation assays

A one-way ANOVA (Dunnett's test) with the pairwise multiple comparison procedure was used to compare the results obtained for the degree of insufflation of the different dry powders with the degree of insufflation of LBG microparticles as reference. All analyses were run using GraphPad Prism® statistical program (GraphPad Software, version 9.4.0.673), and differences were considered significant at a level of $p < 0.05$.

Table 2
Powder appearance, microparticle morphology (by scanning electron microscopy) and particle diameters (Feret and aerodynamic) of the three model dry powder formulations chosen as representative of the whole study. Aerodynamic diameters were calculated using Eq. (1).

Powder: Locust bean gum microparticles		
Feret's diameter: 3.95 ± 4.25 μm **Morphology**		**Aerodynamic diameter:** ≈3.29 μm **Appearance**
		
Powder: Konjac glucomannan/leucine microparticles (KGM/Leu)		
Feret's diameter: 1.18 ± 0.75 μm (Guerreiro, 2015) **Morphology**		**Aerodynamic diameter:** ≈1.40 μm (Guerreiro, 2015) **Appearance**
		
Powder: Dextran sulfate microparticles (DS)		
Feret's diameter: 1.91 ± 1.17 μm (Musacchio and Grenha, 2017) **Morphology**		**Aerodynamic diameter:** ≈1.48 μm **Appearance**
		

2.11.2. For cell viability assays

A two-tailed unpaired *t*-test was performed on the data pertaining to the cell viabilities obtained. Results were analysed in two different scenarios: (1) the influence of insufflation of either air or air plus dry powders and (2) the effect of time after insufflation. All statistical analyses were conducted as above.

3. Results and discussion

3.1. Characterisation of dry powders

A characterisation of relevant physical parameters of three model dry powders chosen as representative (see 3.3.2) for the study described herein, can be seen in Table 2 below.

3.2. Design and development of an insufflation prototype

The aim of the work described in this manuscript was the development of a prototype that enables the insufflation of dry powders. The prototype was concretely conceived in connection with the experimental setup related with cell-based *in vitro* assays in the context of respiratory delivery. Despite the marketed and useful solutions that also permit a similar objective, these constitute an option that is technically complex and expensive. The prototype developed herein intends to provide an alternative and economically viable solution that is affordable for initial stages of formulation development. It should respond to several requirements: i) to be 3D printable, ii) to have dimensions compatible with portability and practical use, iii) to be a simple structure, preferably single-piece and iv) to have a structure enabling weighing of dry powders.

Once the designs of a main body and a matching weighing accessory were finalised (Figs. 1 and 2), and after further adjustments required by the 3D printing process, it was possible to obtain a Technology Readiness Level (TRL) 4 prototype, as shown in Fig. 3 and as thoroughly described in "Portable device to study the exposure of cells to dry powders" (Grenha et al., 2023).

The idea and the design herein presented resulted in a new, economically accessible, and easy to manipulate prototype for *in vitro* insufflation of dry powders.

3.3. Optimisation of the insufflation method

The following step on the development of the prototype would be the definition of the most adequate strategy to insufflate the powders. In brief, an air stream should push the powder off the weighing platform, dragging it down in a homogenous aerosol over a petri dish. After the testing of several tools to blow air into the prototype (lens blower, bicycle pump, syringe, garden spraying device and an air compressor), the latter was the one having the best characteristics for the intended purpose herein described, allowing to maximize the yield of insufflation, calculated using Eq. (3), but using the analytical balance also for weighing the final amount of powder. Afterwards, the optimisation of the insufflation method was performed by applying outlet air pressures between 30 and 60 psi. Overall, pressures of 36 and 42 psi were those maximising the yield of insufflation (determined as above) reaching up to 25 % by weight in average.

Although the results of yield of insufflation are considered low, evidencing unsatisfactory efficiency, the efficacy of the prototype is demonstrated, which is reasonable at this stage of development. Altogether, the optimisation of the insufflation method entails several aspects, one of those being the improvement of the prototype. In fact, a collaboration with a development company is being implemented and a new version of the prototype that replaced the air compressor by a pump pear, among other slight modifications, has permitted an improvement in the yield of insufflation, which is now around 50 %.

Fig. 3. Prototype printed by stereolithography using UV light and a washable resin. On the left, the prototype is assembled, while on the right, both the main body and the weighing head are separated.

3.4. Quartz-crystal microbalance (QCM) assays

3.4.1. Data acquisition

The QCM setup can be seen in Fig. 4.

The frequency counter is one of the crucial parts of the QCM setup, as it reads the oscillation frequency at any given time. For the assessment of the different deposition profiles of the dry powders, it was required that all frequencies during the time of the assay were registered and saved for analysis. This was made possible by a program written in Keysight Visual Engineering Environment (VEE, Pro-version 9.33, Agilent) (see Supplementary Material).

3.4.2. Aerosol characterisation using the QCM

An insufflation method was initially established that required 30 quick trigger activations of the air pistol of the air compressor at an outlet air pressure of either 36 or 42 psi, so that the dry powder would be dragged towards the cell support. However, the first assays using the QCM evidenced its sensitivity towards the insufflations and, thus, further optimisation was required. A first approach relied on the determination of the adequate outlet air pressure, that was changed from 36 psi/42 psi to 30 psi. Moreover, the team believed that the powder would be dragged with fewer activations of the air pistol. A new experimental protocol was devised, in which the minimal number of required insufflations to be applied was assessed, ensuring at the same time the desired frequency change. The stop point would be when the

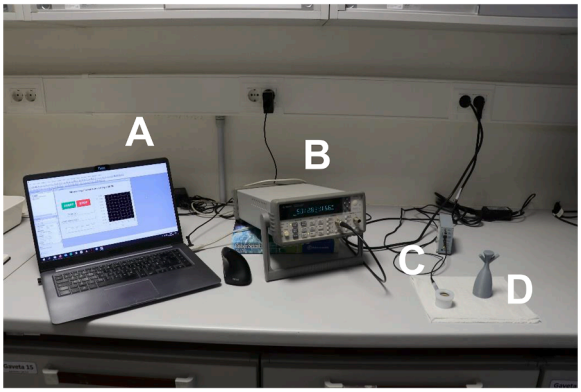


Fig. 4. Quartz Crystal Microabalance (QCM) setup. From left to right: computer with acquisition program (A), frequency counter (B), crystal holder and oscillator (C), and the insufflation prototype (D).

frequency reached a new equilibrium, thus ending the assay. The new method was set at 10 quick trigger activations (or insufflations) of the air pistol at an outlet air pressure of 30 psi, although observations suggesting that the totality of the powder was dispersed after two or three insufflations. In fact, the reduced number of insufflations by comparison with the method defined in Section 2.4, simplified the experimentation and made it easier to apply.

Before using dry powders, the determination of QCM frequency variation in the sequence of air insufflation (control assay) was essential. This was performed with the prototype and the respective variations of frequency are shown in Fig. 5, which shows that, when no powder is used, the frequency remains unaltered.

QCM results are presented for three out of the thirteen microparticle formulations comprised of a diverse set of polysaccharides. The selection was driven by different aerodynamic properties and morphology of the microparticles, which were considered to possibly have an impact on the insufflation process. Furthermore, those differences would enable establishing correlations between the morphology and capacity to disperse. The deposition profile of LBG, KGM/Leu-and DS is shown in Fig. 6.

The QCM works based on a relaxation method. In brief, when the sensor is in equilibrium, but it is disrupted by an external force such as the insufflation of air, the frequency varies, and it only stabilises when this disruption ends. At this point, the QCM resonates to a new equilibrium, thus sensing the mass of dry powder that fell onto it, resulting in a new frequency result. Different dry powders result in different equilibrium frequencies, enabling the establishment of a deposition profile ranking. Of all three dry powders tested, LBG was the one evidencing the highest frequency variation, which translated into a high degree of insufflation (Eq. (4)), thus entailing a good deposition profile. All other remaining powders evidenced a significantly different and low degree of insufflation ($p < 0.05$) when compared to the powders of LBG (Table 3).

Further analysis of the formulation ranking by degree of insufflation and the macroscopic appearance of the tested dry powders permits an organisation of the samples in three different dispersion categories: good, mediocre and bad. Examples of these deposition profiles are shown in Fig. 7, with images of both the petri dish and the QCM crystal.

In brief, the left photographs in Fig. 7 evidence a good powder dispersion, where the petri dish is covered by a homogeneous cloud of dry powder, as well as the QCM crystal. This is a representation of the dispersion pattern of LBG. The middle photographs represent a mediocre powder dispersion, where the petri dish has areas covered by dry powder and other areas where agglomerates are visible. The photograph evidences dry powder concentrated in one region of the crystal, as representative of the dispersion pattern of KGM/Leu. The photographs on the right correspond to bad powder dispersion, where a few particles are seen spread on the petri dish and on the QCM. In this latter case, an area with dry powder homogenously dispersed is absent and it is representative of the DS powder.

Despite the relatively low efficiency of the prototype (which is currently being addressed as stated above), the major objective of developing a device that allows the insufflation of dry powders over a specific support was successfully accomplished, regardless of the different characteristics of the samples used. Furthermore, it was also observed that the first burst of air removed almost all the powder out of the weighing head platform. Despite that, when the QCM is being used, the change in the frequency is always detected.

A deeper analysis of all data concerning the dry powders used (morphology, deposition profile, degree of insufflation, and overall aspect of the powders) suggests that a mixture of both spherical and irregular shaped particles favours insufflation in relation to only spherical particles. Completely spherical particles lead to inadequate patterns of deposition as observed for DS microparticles. This is in accordance with general observations of better aerodynamic behaviour for irregularly-shaped particles, as the surface area that contacts with air is higher than that of spherical particles (Yaqoubi et al., 2021).

In all, the inclusion of the QCM in the experimental setting herein described constitutes a more complete approach of the *in vitro* analysis of dry powders presenting more advantages than a typical analytical balance. The use of the QCM technology allows following the insufflation process in detail through frequency change and describe the behaviour of the dry powder, as well as its deposition profile, as shown in Figs. 5 and 6. On the contrary, using an analytical balance to perform this type of analysis results in interpretations based solely on a single measurement, the amount of the dry powder that falls into a specific support (Eq. (3)). Therefore, the use of the analytical balance ignores the physical properties of the dry powder that is being tested, as well as its ability to adequately disperse in the support, which may generate misinterpretation of results. In contrast, data generated by the QCM provides more comprehensive information, thus supporting better decisions.

3.4.3. Cell viability after insufflation

The main goal of developing the prototype was to enable the delivery of dry powders onto cell surfaces, aiming at simulating more appropriately the process of inhalation. In order to provide a proof-of-concept of the use of the prototype for the proper objective, the viability of respiratory cells was determined after insufflation of LBG microparticles (LBG). Cell viability is a very common parameter characterised in the context of the toxicological evaluation of materials, being described in international guidelines as a measurement of the toxicity of an API towards cells (ISO 10993-5-2009 2009). A549 cells were selected as model of the respiratory epithelium, because of their widespread use in this context.

On insufflating dry powders over cells in culture using the prototype, an air stream is applied to the cell layer. Depending on its intensity, a damaging effect could be induced. Therefore, it was deemed important to determine the impact of that air stream on cell viability. A control assay was, thus, performed consisting only of air insufflation over cells.

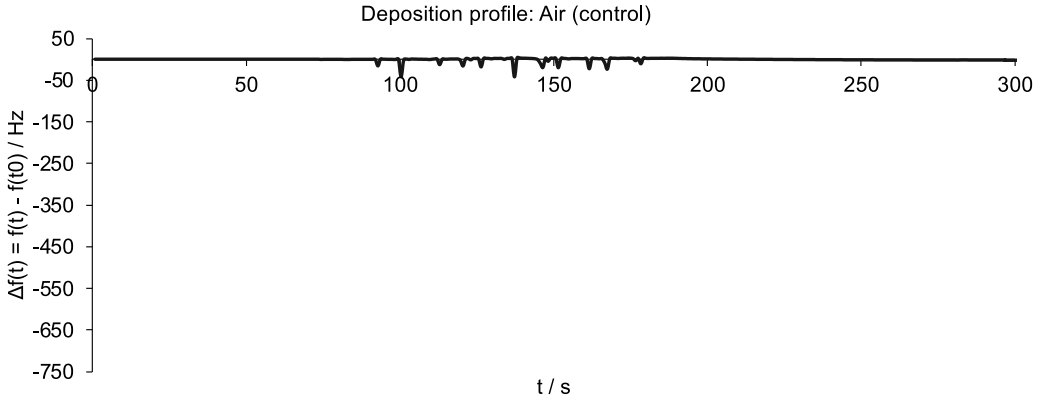


Fig. 5. Variation of frequency of the QCM upon insufflation of air into the prototype (no powder present). Results are presented as mean ± SD, $n = 6$.

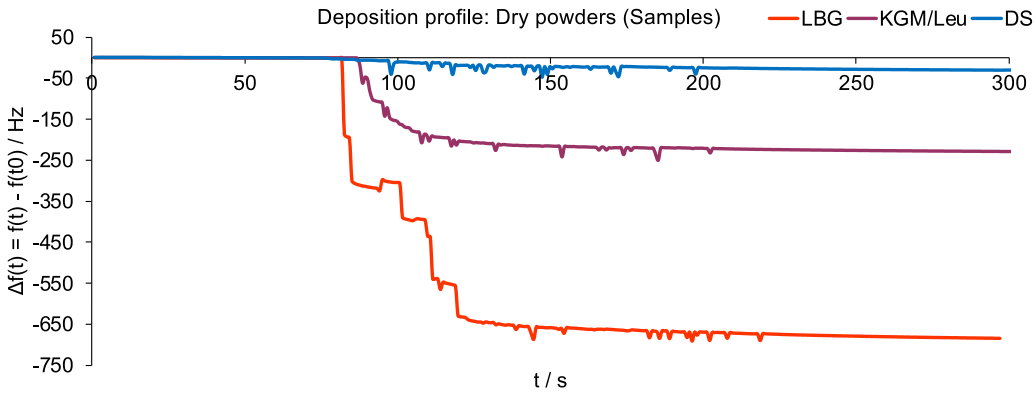


Fig. 6. Deposition profile for locust bean gum (LBG), konjac glucomannan/leucine (KGM/Leu) and dextran sulfate (DS) microparticles. Results are presented as mean ± SD, $n = 6$.

Table 3
Degree of insufflation of the selected dry powder formulations and ranking of performances according to the results. Results are presented as mean ± SD, $n = 6$.

Dry powders	Degree of insufflation	Deposition profile
LBG	100	Good
KGM/Leu	33.98 ± 8.26	Mediocre
DS	4.23 ± 1.44	Bad

DS: dextran sulfate; KGM konjac glucomannan; LBG: locust bean gum; Leu: l-leucine.

The results of air insufflation and exposure to LBG microparticles are displayed in Fig. 8.

The assay devised to resemble the condition of pulmonary inhalation, so cells were cultured in petri dishes, and cell culture medium removed prior to the insufflation of either air or dry powder. A small volume of cell culture medium (500 μL) was afterwards added to avoid cell desiccation during the exposure time. Overall, as observed in Fig. 8, the insufflation of air and of dry powders did not impact cell viability to result in a major cell death event. In fact, cell viabilities roughly above 70 % were always determined, indicating absence of cytotoxic effect. Nevertheless, the insufflation of air, used herein as control, induced a lower reduction in cell viability than the insufflation of microparticles. The first indication on the used method of insufflation and on the impact of LBG microparticles corresponds to a safe profile.

4. Conclusions

A prototype for dry powder insufflation was designed and developed, using 3D printing methods for its production. When compared with similar devices on the market it is simpler to use, more versatile, and much less expensive. It permits the homogenous dispersion of the insufflated powders over a petri dish and, when coupled with a QCM device, enabled a more detailed study on how dry powders disperse over the supports. The prototype still requires further improvement, especially regarding the method for powder weighing and the efficiency of insufflation. A company was recently subcontracted to provide advancements in the optimisation of the prototype. The intellectual property of the prototype is already protected, in the form of an international patent application currently under examination. In parallel, the impact of insufflation of air and of LBG microparticles was assessed, and it was observed that both did not impact cell viability. In fact, cell viabilities roughly above 70 % were always determined, which indicates the absence of cytotoxic effect. Next stages of experimentation envisage the culture of cells over the crystal of QCM for an evaluation of particle/cell interaction upon insufflation of the dry powders. The inclusion of this technology in experimentation, especially concerning the *in vitro* analysis of dry powders, can provide more information and data in formulation development, further complementing analytical procedures and the strengthening of results and conclusions.

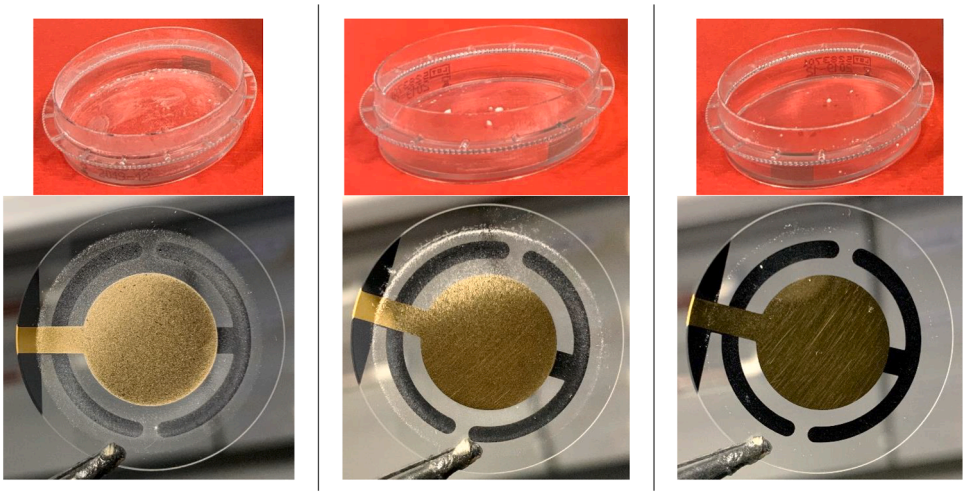


Fig. 7. Examples of deposition profiles in a petri dish (proof-of-concept assays) and in the QCM (insufflation assays). From left to right: good, mediocre, and bad deposition profiles.

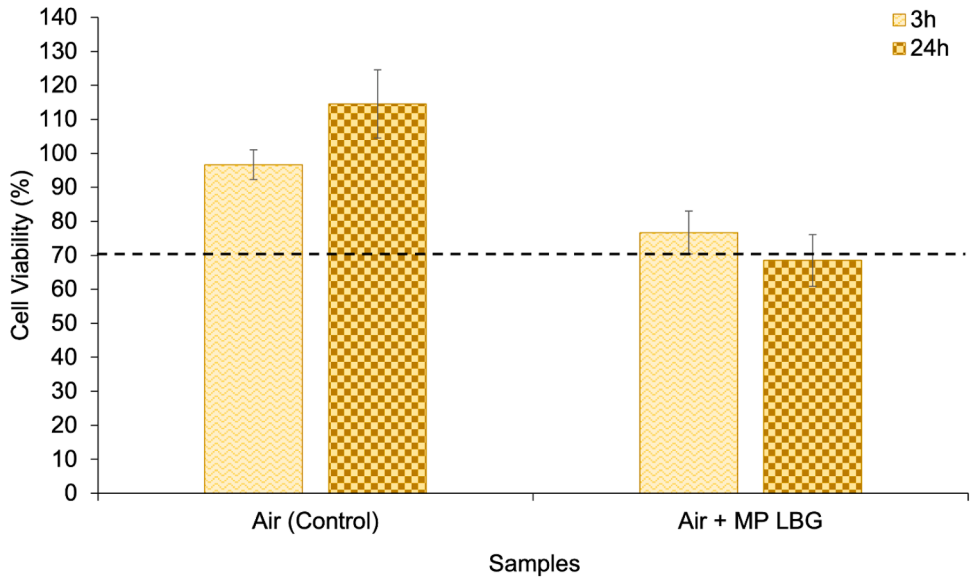


Fig. 8. Viability of A549 cells upon 3 h and 24 h exposure (textured columns in yellow and brown) to air (control) and a dry powder comprised of locust bean gum (LBG) microparticles (MP). Data represent mean ± SEM (n = 3). Dashed line represents 70 % cell viability.

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CRediT authorship contribution statement

Jorge F. Pontes: Writing – original draft, Methodology, Investigation. **Hermínio P. Diogo:** Methodology. **Eusébio Conceição:** Validation, Methodology, Conceptualization. **Maria P. Almeida:** Investigation. **Rui M. Borges dos Santos:** Writing – review & editing, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Ana Grenha:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2024.106775.

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