Respiratory Medicine 153 (2019) 52–59

Contents lists available at ScienceDirect

Respiratory Medicine

journal homepage: www.elsevier.com/locate/rmed

Influence of frequency and amplitude on the mucus viscoelasticity of the novel mechano-acoustic Frequencer™

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ARTICLE INFO

Keywords:
Cystic fibrosis
Frequencer™
Saline solution
Mechano–acoustic vibration
Infrasound
Frequency
Amplitude

ABSTRACT

Background: Cystic fibrosis affects 1/3200 Caucasians. This genetic disease disturbs the ion and water homeostasis across epithelia, thus rendering mucus more viscous and harder to expel. Conventional treatments rely on the clapping method coupled with postural drainage. Despite the effectiveness of these procedures, they are invasive and enervating.

Methods: Here we study a new mechano–acoustic treatment device to help patients expectorate excess mucus, the Frequencer™. We test both normal and pathological synthetic mucin solutions (1 % and 4 % by weight) *in vitro*. We varied the frequency applied (from 20 Hz to 60 Hz) as well as the amplitude (from 50 % to 100 % intensity). Moreover, we assessed the effect of NaCl on mucus rehydration.

Results: A frequency of 40 Hz coupled with a 0.5 gL^{-1} NaCl solution provokes partial mucus rehydration, regardless of the amplitude selected, as the work of adhesion measurements evidenced.

Conclusions: Mechanical solicitation is fundamental to help patients affected by cystic fibrosis expectorate mucus. With an operating frequency of 20 Hz to 65 Hz, the Frequencer™ provides a gentler therapy than traditional methods (conventional chest physiotherapy). The Frequencer™ proved to be effective in the homogenization of synthetic mucin solutions *in vitro* in 20 min and elicited improved effectiveness in a mucin-rich environment.

1. Introduction

Cystic fibrosis (CF) is one of the most common life-threatening genetic pathologies affecting Caucasians, with an estimated average incidence equal to 1/3200. Ireland reports the highest CF occurrence with an incidence of 1/1800, whereas Finland CF incidence hits 1/25000 [1]. In Canada, between 1985 and 1989, the median survival age was 36.7 y for males and 27.8 y for females. Nowadays it has increased to 36.8 and 48.5 y for both genders in Canada and US, respectively [2].

CF affects epithelia and exocrine glands. This monogenic and autosomal recessive condition results from the absence or malfunction of the gene that encodes for the cystic fibrosis transmembrane conductance regulator (CFTR), identified on the long arm of chromosome 7 in 1989 by Rommens et al. [3–5]. The CFTR protein is found in various epithelial cell types, including respiratory epithelia and submucosal glands. It acts as cAMP-mediated chloride channel that regulates the ion and water homeostasis across epithelia [6,7], which affects the hydration and conservation of rheological properties of mucus.

Mucus is a heterogeneous, adhesive and viscoelastic reversible gel [8] composed of water, globular proteins, carbohydrates, lipids, salt and water, and antimicrobial factors [9]. Mucins, namely large peptidoglycan biopolymers, are the main constituents of mucus. They provide the structural framework of the mucus layer and are responsible for its viscoelasticity [10]. The CFTR channels dysfunction determines an excessive trans-epithelial water absorption. Mucus dehydration is accompanied by the alteration of the levels of reduced glutathione and myeloperoxidase, and of the pH. This provokes the formation of additional inter-chain mucin bond thus increasing the viscoelasticity of mucus, reducing its viscosity and impeding its transport throughout the airways [11,12].

In healthy humans, the mucin content is 1 % by weight [13] with a total protein and glycoprotein concentration of about 2.5 %. This value increases up to (5.6 ± 2.0) % by weight for patients affected by CF [14]. These factors determine abnormal and non-optimal physical and

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https://doi.org/10.1016/j.rmed.2019.04.011
Received 1 February 2019; Received in revised form 9 April 2019; Accepted 9 April 2019
Available online 27 May 2019
0954-6111/

rheological characteristics, and rather than protecting the host, they provide an environment wherein the pathogen is protected and able to proliferate [10], thus entailing a chronic lung infection.

The bacterial colonization is almost impossible to eradicate and it is responsible for the morbidity and mortality of the disease [15]. Therefore, the pulmonary mucus clearance represents a key defence mechanism to protect the lung against inhaled pathogens and particles.

Therapeutic interventions, which aim at restoring the mucociliary and cough clearance, correct the rheological and physical abnormalities of mucus through rehydration, lubrication and fluidification. Elkins et al. reported that hypertonic saline aerosol improves lung functions with a significant reduction of pulmonary exacerbations [16]. Salt deposits onto the surface of the liquid layer and osmotically draws additional water to it, thus rehydrating mucins and restoring the mucociliary clearance [17]. This creates an environment that is not suitable for microbial growth and the severity of inflammation diminishes [18]. Tabatabaei et al. also reported an improvement of the forced expiratory volume in 1 s (FEV1) in 44 children (mean age of 11 years) upon the inhalation of a 5 % NaCl solution over a period of one year [19]. Although, anti-inflammatory [20], enzyme and gene therapies [21,22], antibiotics [23] and bronchodilators have been optimized in the last 30 years [24,25], they still require complementary mechanical procedures for an effective bronchial drainage. Acoustic percussion has been suggested as a potential technique to help induce airway clearance [12,26] as mucin–rich gels show enhanced viscoplasticity and shear–thinning ability [27]. In fact, mucus' stress-strain curve is not linear [28] and its elasticity changes with the stress application rate and a continuous stress is needed to induce airway clearance. Button et al. [29] theorized that exercise, chest percussion and the use of mechanical therapeutic devices increase ATP release, which in turn promotes Cl^- secretion and further mucus rehydration. The foremost non-pharmacological approach is the conventional chest physiotherapy ('clapping method', CCPT) coupled with postural drainage [30].

Even though CCPT is effective, it requires a great deal of time and work, both for inpatients and outpatients with compromised airway clearance. As a result, many patients refuse to perform their daily physiotherapy and interrupt it with harmful consequences. Smarter and less invasive solutions have been explored in recent years. Silva et Monnin compared the effects that eight airway clearance techniques (including postural drainage) have on bronchiectasis and on the clearing of bronchi. Oscillating techniques surely improve the quality of life, reduce the symptoms related to bronchial obstruction, increase the amount of mucus expectorated with cough, and reduce dynamic hyperinflation. However, the data available is not comprehensive enough to elucidate the validity of such therapies [31,32]. In fact, there is no statistical difference among the most widespread airway clearance regimens over one year (ACBT, AD, Cornet, Flutter and PEP) [33].

Mr. Louis Plante, a Québec origin patient affected by CF, invented a device able to generate mechanical and acoustical vibrations at low frequency [34], the Frequencer™. Mr. Plante founded the company Dymedso in 2002 together with Mr. Yvon Robert to commercialize the Frequencer™. It transmits sinusoidal mechanical and acoustical vibrations to the airways within the chest. The adjustable frequency provokes sympathetic resonance within the thorax and it induces a cough, followed by the expectoration of sputum [34].

Cantin et al. [26] described the operation of the Frequencer ™ and compared it to conventional chest physiotherapy techniques CCPT, using the expectorated sputum weight as the main outcome measurement. They reported that both treatments were equally effective and that the Frequencer™ offered significant benefits over the CCPT, including a reduction in side effects, autonomy and ease of use for many patients [26]. In addition to the reduction of mucus viscosity through repetitive vibrations, the Frequencer™ may also induce the shearing at the mucus/airway interface, thus reducing mucus adhesion through mechanical and acoustical waves coupling. Patients using the Frequencer™ have reported easier mucus clearance and expectoration at specific frequencies from 37 Hz to 42 Hz. The mechanisms by which the Frequencer™ helps mobilize bronchial secretions are not fully known.

On purpose, we investigated how mucus viscoelasticity and hydration change depending on the frequency and amplitude delivered by the Frequencer™ *in vitro*. Specifically, the lack of mucus fluidity in patients with CF is ascribable to its dehydration as a consequence of insufficient excretion of NaCl to the airways, leading to the increase of secretion viscosity [35]. We also analysed how the NaCl content affects mucus fluidification upon the application of the Frequencer™ at different frequencies and amplitudes.

Although the treatment with 0.9 % isotonic saline and 3.0 % and 7.0 % hypertonic salines inhaled twice per day for 48 days proved to be effective in the improvement of lung function [36], the NaCl concentration of blood serum and tissues is about 0.1% [37] and 0.15 %1.0 % [38], respectively. Moreover, the 0.9 % isotonic saline contains 10 % more Na^+ and 50 % more Cl^- than human serum [37]. Therefore, we studied the effect of a 0.5 gL^{-1} NaCl solution, which corresponds to a 0.1 % NaCl solution treatment, to assess the effect of the Frequencer™ in physiological blood serum conditions. Further investigations will disclose the effect of the device when using 0.9 %, 3.0 % and 7.0 % salines.

2. Methods

2.1. Material

Mucin from porcine stomach Type II (Sigma Aldrich) simulated human mucus. We prepared mucin solutions by suspending 1 % by weight of mucins (healthy person, S1) and 4 % by weight mucins (person affected by CF, S2) in distilled water. A magnetic stirrer vigorously agitated the solution overnight.

2.2. Frequencer™ and reactor description

The device comprises a main unit (a) and a treatment interface (b) (Fig. 1). The latter couples an acoustic transducer to an acoustic coupling chamber in a single casing.

The main unit includes a digital user interface to regulate frequency and intensity of the treatment. The generated frequency goes from a minimum of 20 Hz to a maximum of 65 Hz, while the sound pressure output can be adjusted from 0 psi to 0.4 psi. The Frequencer™ accessories include four different sized adapters ((c) in Fig. 1) corresponding to different users (baby, child, young adults, and adults). The adapters differ for what concerns the size of the opening, corresponding to the area of treatment. All our experiments were carried out with the adapters for children, which has an opening of 55 mm. Each treatment with the Frequencer™ consists in the application of the transducer to 6 different areas of the chest from 23 min for each zone. The reactor for the experiments was a cylindrical plastic reactor (ID = 46 mm, H = 44 mm, capacity of 66 mL) with an external diameter corresponding to the size of the opening of the adapter ((d) in Fig. 1).

2.3. Frequencer™ experiments

We designed 48 experiments to assess the effect of three main parameters (Table 2).

In a typical experiment, we filled the reactor with either the solutions of mucus (blank tests, 66 mL) or the solutions of mucus (33 mL) in contact with either water or brine (33 mL) (Table 1). In the latter case, we filled the reactor with equal amounts of mucus solution, always at the bottom, and water or NaCl solution, without stirring the mixture. We prepared fresh mucus solutions before each test.

We covered the reactor with a paraffinic film to simulate the presence of dermis. An adhesive paste held it on position. We applied the adapter (MOLD150) on top of the reactor. After 20 min at room temperature (25 °C), we collected mucus both from the top (T samples) and

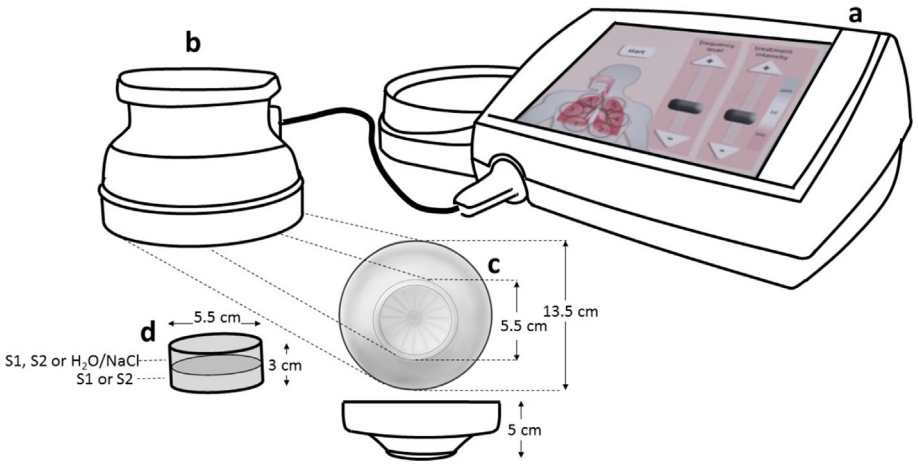

Fig. 1. Experimental set-up with the Frequencer™.

Table 1
Experimental parameters varied and their set values.

Frequency (Hz)	Amplitude (%)	Composition
20	50	Mucus
40	100	Mucus + Water
60		Mucus + NaCl solution (0.5 gL^{-1})
		Mucus + NaCl solution (saturated)

the bottom (B samples) of the reactor. We selected 20 min as a duration for the experiments because a typical treatment with the Frequencer™ consists in the application of its head for 5 min on each quarter of the chest, for a total of 20 min.

2.4. Analytical

An ANTON PAAR DMA 4500 determined the density of T and B samples. It measures the damping of a U-tube's oscillation caused by the viscosity of the filled-in sample and checks it against a reference oscillator. Two integrated Pt 100 platinum thermometers accurately controlled the temperature. We determined all density values at 20 °C.

A THERMO Scientific HAAKE iQ air viscotester (coaxial cylinder configuration) measured the viscosities of the samples. We introduced 3 mL of sample in the instrument chamber, then we waited 5 min to equilibrate a temperature of (25.00 ± 0.02) °C. We set a shear rate of 100 s^{-1} for 1 min and then the instrument raised it to 500 s^{-1} over 5 min. We regressed the data and the best fit was obtained with the Carreau–Yasuda model, which also fits the rheological behavior of human cervical mucus [39].

A METTLER TOLEDO coulometric Karl Fischer titrator (model V20S) measured the water content in T and B samples. Differences in water composition between T and B of a single test may point out an effect elicited by the Frequencer™. We used a one-component Karl-Fischer reagent. The titrant contains iodine, sulfur dioxide and imidazole, dissolved in a suitable alcohol (HYDRANAL ™ Composite 5, Honeywell). The solvent is dry methanol (HYDRANAL™, Honeywell). We diluted each sample in methanol and injected few drops (about 80 mg) of such diluted samples. The water content obtained (% by weight) was then resized to the original sample by simple mass balance. We measured at least two times the water content of each sample.

We monitored the salt concentration via colorimetric Mohr titration. However, we could not clearly see the end-point of the titration. This is due to the fact that mucins have cysteine-rich domains and $AgNO_3$ complexes with the sulfur present on such amino acid [40]. Therefore, we could not precisely detect the end-point of the titration since the color change was gradual. Hence, we measured the conductivity of the sample upon the addition of $AgNO_3$ (0.1 mol L^{-1}, Honeywell) with a HANNA HI763100 conductivity meter. The conductivity decreases linearly as long as Ag^+ complexes Cl^-. Successively, after the complete precipitation of chloride ions, the conductivity of the solution increases linearly with the addition of extra Ag^+ ions. The end-point is given by the intersection of the two lines.

A DataPhysics OCA tensiometer measured the contact angle (CA) and the surface tension (ST) for each test. From these primary data, we calculated the work of adhesion (WA) of the sample [41]. We measured CAs at 20 °C and on a solid glass flat surface. Despite glass is not representative of the internal bronchial airways, by running all the analyses on the same surface we could discern diverse behaviours. We analyzed all T and B fractions, except for viscosity, which was determined for random spot-checks (6 samples, 12 T and B).

2.5. – Statistical analysis

We calculated the theoretical error of the water content measurement applying the formula for the error propagation assuming independent variables (Eq (1)).

$$\Delta f = \sqrt{\left(\frac{\partial f}{\partial x}*\Delta x\right)^2 + \left(\frac{\partial f}{\partial y}*\Delta y\right)^2 + \left(\frac{\partial f}{\partial z}*\Delta z\right)^2} \quad (1)$$

in which x, y, z, etc. are the function variables (mass of samples, volumes, water content of the solvent, etc.) and Δi is the uncertainty of the ith variable or the function (f). We applied Eq. (1) to all T and B samples, to determine if the two samples had a significant difference. We applied the t–test of the means to understand whether the averages of contact angles obtained from the samples prepared at diverse frequencies and amplitudes were statistically equivalent (null hypothesis) or different for a 95 % confidence level.

3. Results

3.1. Water repartition and mucus wettability

For some analyses the water content was higher than 100 % with a relative error considerably high for some samples (10 % to 20%) (Table 2). This is ascribable to the nature of the Karl-Fischer method, which is more suitable for lower water concentrations. Moreover, upon the dilution of samples in methanol, we observed the denaturation of proteins and their precipitation, which altered titration results. For these reasons, we decided not to take into account the results of the Karl-Fischer titration, as they are not reliable. Hence, we applied the t-Student calculation to the contact angle (CA) values obtained for all T and B fractions to discern significant differences between top (T) and

Table 2
Water content in top (T) and bottom (B) samples and absolute theoretical error (Eq. (1)). S1 = 1 % by weight solution of mucins; S2 = 4 % by weight solution of mucins (person affected by CF, S2) in distilled water. I = Intensity of the maximum output pressure of the Frequencer™.

Test	Sample	H_2O (%)	Δf (±)	Test	Sample	H_2O (%)	Δf (±)
M1 T	S1 20 Hz, 50 % I	95	8	M25 T	S2 20 Hz, 50 % I	96	4
M1 B		108	9	M25 B		96	6
M2 T	S1 20 Hz, 100 % I	109	20	M26 T	S2 20 Hz, 100 % I	100	4
M2 B		75	3	M26 B		89	15
M3 T	S1 40 Hz, 50 % I	98	22	M27 T	S2 40 Hz, 50 % I	89	8
M3 B		97	4	M27 B		106	8
M4 T	S1 40 Hz, 100 % I	96	2	M28 T	S2 40 Hz, 100 % I	105	91
M4 B		93	3	M28 B		83	89
M5 T	S1 60 Hz, 50 % I	97	9	M29 T	S2 60 Hz, 50 % I	109	2
M5 B		99	26	M29 B		96	20
M6 T	S1 60 Hz, 100 % I	98	2	M30 T	S2 60 Hz, 100 % I	107	13
M6 B		98	3	M30 B		93	3
M7 T	S1 + H_2O 20 Hz, 50 % I	96	1	M31 T	S2 + H_2O 20 Hz, 500 % I	98	17
M7 B		99	0	M31 B		63	13
M8 T	S1 + H_2O 20 Hz, 100 % I	97	3	M32 T	S2 + H_2O 20 Hz, 100 % I	97	14
M8 B		99	9	M32 B		99	1
M9 T	S1 + H_2O 40 Hz, 50 % I	99	3	M33 T	S2 + H_2O 40 Hz, 50 % I	105	2
M9 B		97	15	M33 B		96	7
M10 T	S1 + H_2O 40 Hz, 100 % I	101	4	M34 T	S2 + H_2O 40 Hz, 100 % I	98	14
M10 B		101	5	M34 B		99	12
M11 T	S1 + H_2O 60 Hz, 50 % I	94	30	M35 T	S2 + H_2O 60 Hz, 50 % I	98	4
M11 B		97	16	M35 B		93	10
M12 T	S1 + H_2O 60 Hz, 100 % I	95	13	M36 T	S2 + H_2O 60 Hz, 100 % I	100	7
M12 B		96	1	M36 B		101	2
M13 T	S1 + brine 20 Hz, 50 % I	89	6	M37 T	S2 + brine 20 Hz, 50 % I	102	36
M13 B		82	53	M37 B		79	5
M14 T	S1 + brine 20 Hz, 100 % I	92	10	M38 T	S2 + brine 20 Hz, 100 % I	81	19
M14 B		90	13	M38 B		80	22
M15 T	S1 + brine 40 Hz, 50 % I	90	10	M39 T	S2 + brine 40 Hz, 50 % I	72	71
M15 B		89	1	M39 B		77	6
M16 T	S1 + brine 40 Hz, 100 % I	91	10	M40 T	S2 + brine 40 Hz, 100 % I	63	15
M16 B		84	9	M40 B		106	30
M17 T	S1 + brine 60 Hz, 50 % I	89	3	M41 T	S2 + brine 60 Hz, 50 % I	85	20
M17 B		88	9	M41 B		83	6
M18 T	S1 + brine 60 Hz, 100 % I	90	9	M42 T	S2 + brine 60 Hz, 100 % I	85	9
M18 B		88	4	M42 B		78	12
M19 T	S1 + NaCl (0.5 gL^{-1}) 20 Hz, 50 % I	98	8	M43 T	S2 + NaCl (0.5 gL^{-1})20 Hz, 50 % I	94	3
M19 B		99	8	M43 B		93	5
M20 T	S1 + NaCl (0.5 gL^{-1}) 20 Hz, 100 % I	99	16	M44 T	S2 + NaCl (0.5 gL^{-1})20 Hz, 100 % I	91	4
M20 B		100	2	M44 B		92	1
M21 T	S1 + NaCl (0.5 gL^{-1}) 40 Hz, 50 % I	100	1	M45 T	S2 + NaCl (0.5 gL^{-1}) 40 Hz, 50 % I	93	1
M21 B		99	2	M45 B		92	4
M22 T	S1 + NaCl (0.5 gL^{-1}) 40 Hz, 100 % I	99	2	M46 T	S2 + NaCl (0.5 gL^{-1}) 40 Hz, 100 % I	93	1
M22 B		101	1	M46 B		92	9
M23 T	S1 + NaCl (0.5 gL^{-1}) 60 Hz, 50 % I	100	5	M47 T	S2 + NaCl (0.5 gL^{-1}) 60 Hz, 50 % I	93	1
M23 B		100	5	M47 B		93	0
M24 T	S1 + NaCl (0.5 gL^{-1}) 60 Hz, 100 % I	102	2	M48 T	S2 + NaCl (0.5 gL^{-1}) 60 Hz, 100 % I	89	32
M24 B		104	1	M48 B		92	4

Table 3
Frequencies and amplitudes that provoke significantly different contact angles (CAs) between T and B fractions of a single test. The symbol "+" indicates when the null hypothesis (T = B) was rejected.

	Differences between top (T) and bottom (B) – Contact angles					
Frequency	20 Hz		40 Hz		60 Hz	
Intensity	50 %	100 %	50 %	100 %	50 %	100 %
S1 (1 % mucin)	+	+		+		
Water	+		+			
Saturated (brine) 0.5 gL^{-1}	+	+	+	+	+	
S2 (4 % mucin)				+	+	+
Water					+	
Saturated (brine) 0.5 gL^{-1}	+	+	+			+
						+

bottom (B) samples for each test (Table 3).

CA values ranged between 20° and 65°. We registered both left and right CA and we averaged them because these values were always similar (less than 0.9° difference). Specifically, on the S1 solution (mucus of a healthy person), the Frequencer™ causes a repartition between the T and B fraction that is more evident at the lower frequencies of 20 Hz and 40 Hz. At 60 Hz (both at 50 % and 100 % amplitudes), infrasound homogenises the solution and, as a result, there is no difference

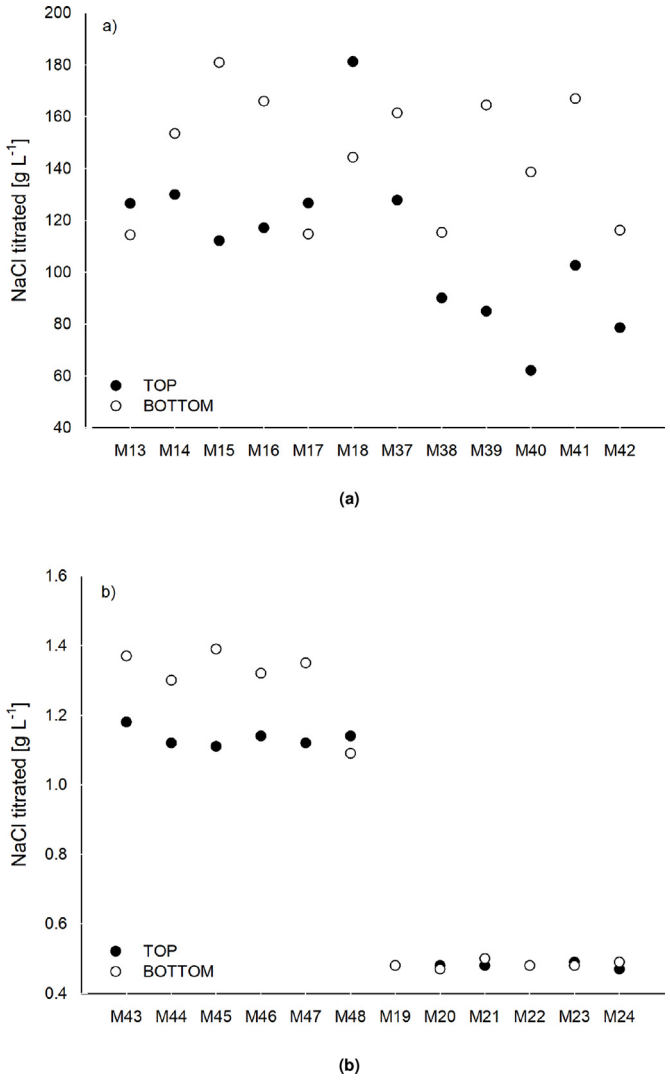

Fig. 2. Na^+ content in top (water rich) and bottom (mucin rich) fractions determined by conductimetric analysis. M1–12 and M25–36 are not reported because those were the tests with only mucin solutions (S1 and S2) and water. M13–M18: mucin S1 (healthy) + brine; M19–20 mucin S1 (healthy) + 0.5 gL^{-1} NaCl; M37–M42 mucin S2 (thick) + brine; M43–M48 mucin S2 (thick) + 0.5 gL^{-1} NaCl

between T and B samples. The same happens when S1 is in contact with pure water: either higher frequency (60 Hz) or pressure (100 %) are required to homogenise the sample. When in contact with a water solution saturated with NaCl, the mucus sediments at the bottom of the reactor at all conditions, except for the highest frequency and pressure (sample M18).

Unsurprisingly, this sample was the one with the highest content of NaCl in the T fraction (Fig. 2), which translates in a higher water content in the same sample. Samples of S1 in contact with 0.5 gL^{-1} of NaCl under the action of the Frequencer™ (tests M19-M24) do not show significant differences. The NaCl concentration is in fact the same for the T and B fractions (Fig. 2b). An equal repartition of the salt between the two phases likely translates in the same repartition of water between the two phases for less concentrated mucin solutions (S1).

The wettability behaviour of S2 samples (mucus of a patient affected by CF) rather follows an opposite and an unpredictable trend. Frequencies of 20 Hz and 40 Hz homogenise the solution of S2 (tests M25-M28), whereas 60 Hz induce a partition of mucus between T and B (tests M29, M30). In the presence of additional water (M31-M34), we can consider that all frequencies and powers homogenise the samples.

Table 4
Parameters of the Carreau-Yasuda equation regressed from the experimental data and calculated viscosities at 100 Hz and 500 Hz $\chi^2 < 0.01$ for all samples.

Sample	Carreau–Yasuda parameters				Viscosity (Pa s)	
	η_{00} (Pa s)	η_0 (Pa s)	$\dot{\gamma}_\beta$ (Hz)	n	$\eta_{fit\dot{\gamma}=100\,Hz}$	$\eta_{fit\dot{\gamma}=500Hz}$
M15 B	1.78	1.89	316.5	2.92	1.86	1.78
M15 T	1.20	11.52	1.9	0.23	2.83	1.97
M18 B	1.70	1.88	97.8	0.74	1.81	1.72
M18 T	1.73	2.77	85.1	0.71	2.29	1.81
M36 B	2.05	5.12	50.2	0.16	4.44	3.55
M36 T	3.92	3.34	443.8	2.12	3.40	3.82
M37 B	2.63	4.34	166.8	0.18	4.24	3.75
M37 T	4.56	6.46	211.9	0.51	6.27	5.28
M42 B	3.27	4.49	212.4	0.71	4.33	3.60
M42 T	4.65	5.60	2632.0	56.09	5.53	4.78
M48 B	3.79	4.82	381.2	1.64	4.71	3.99
M48 T	2.94	3.55	283.1	1.66	3.44	3.00

In the presence of salt, either as a saturated solution (tests M37-M42) or in the concentration of 0.5 gL^{-1} (tests M43-M48), the wettability behaviour is unclear.

3.2. NaCl *repartition*

The conductimetric analyses of the samples containing the 0.5 gL^{-1} NaCl solution confirm the trend obtained from the CA statistical comparative analysis. In fact, the salt concentration is mostly constant for T and B fractions (Fig. 2).

3.3. *Rheological analysis and fraction densities*

The viscosity of T and B samples of tests with brine is lower than the viscosity of samples containing water (Table 4, see M 37 and M 42). Density values confirm that the Frequencer™ elicits the sedimentation of mucus at the bottom of the reactor at 60 Hz and 100 % amplitude: the density of T samples is smaller than that of B samples, 1.0007 g cm^{-3} and 1.1144 g cm^{-3}, respectively (Table 5).

3.4. *Work of adhesion*

WAs for S2 are generally lower than S1's (Fig. 3) and are independent from amplitude over the frequencies tested. This observation is valid considering salt concentration (Fig. 4). The proposed experimental design analyses the work of adhesion (WA) with respect to four factors: mucin mass fraction (*M*, two levels, 1 % and 4 %), salt concentration (*S*, three levels, 0 gL^{-1}, 0.5 gL^{-1} and 360 gL^{-1}),

Table 5
Density values at 20°C.

Composition	Test	Fraction	Density (g cm^{-3})
S1	M1–M6	T	1.0001
		B	1.0001
S1 + Water	M7–M12	T	0.9999
		B	0.9999
S1 + Brine	M13–M18	T	1.0008
		B	1.0008
S1 + 0.5 gL^{-1} NaCl	M19–M24	T	0.9999
		B	1.0001
S2	M25–M30	T	1.0011
		B	1.0011
S2 + Water	M31–M36	T	1.0005
		B	1.0005
S2 + Brine	M37–M42	T	1.0007
		B	1.1144
S2 + 0.5 gL^{-1} NaCl	M43–M48	T	1.0005
		B	1.0005

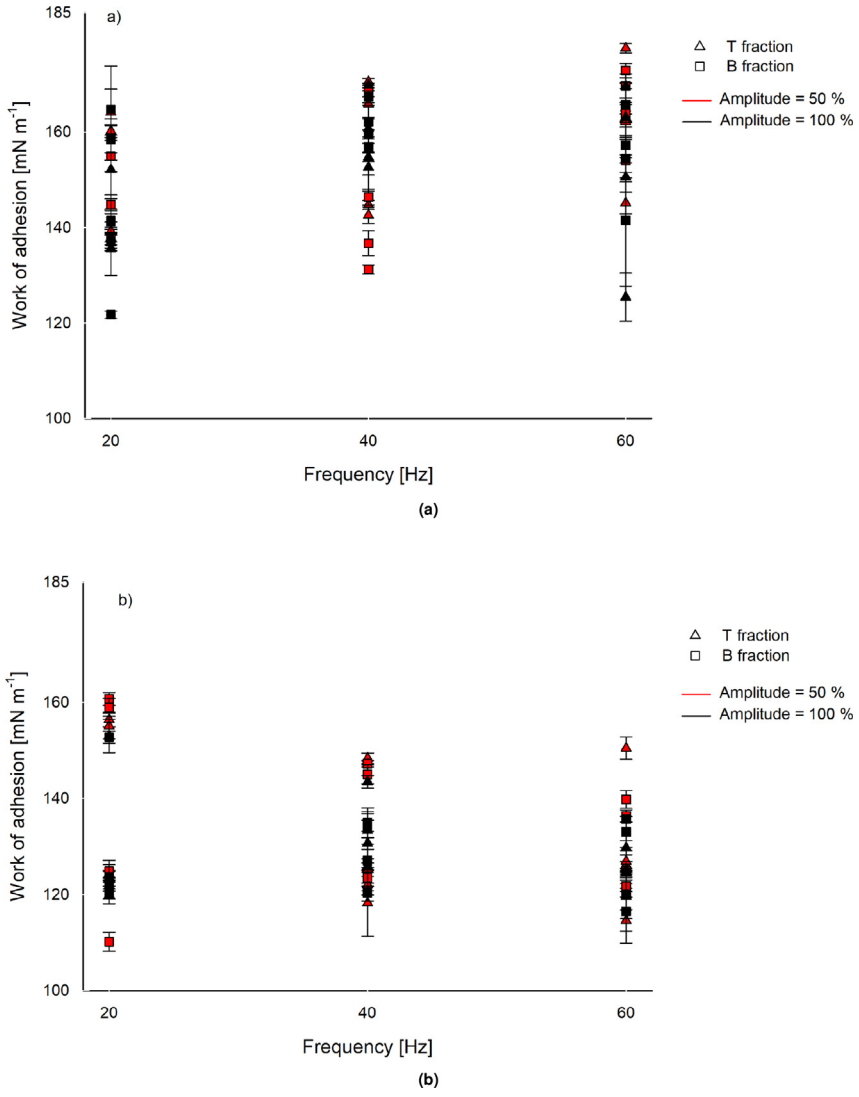

Fig. 3. Work of adhesion for S1 (a) and S2 (b) solutions at 50 % (red) and 100 % (black) amplitudes. Triangles represent T fraction and squares B fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

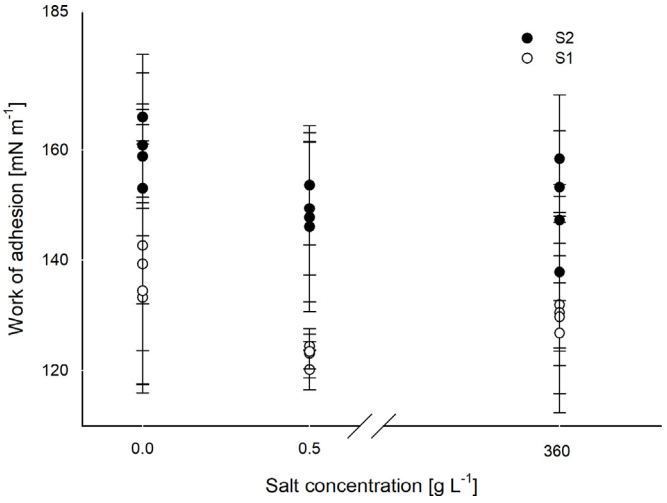

Fig. 4. Work of adhesion of S1 (white) and S2 (black) versus salt concentration.

Frequencer™ operational frequency (*F*, three levels, 20 Hz, 40 Hz and 60 Hz) and amplitude (*A*, two levels, 50 % and 100 % with respect to the maximum output). A full factorial regression identifies the main effects from the rest of the negligible interactions: the model fits the data with an 18 parameters equation and a R^2 of 74 % (squared cross-product interactions are neglected). However, this full factorial regression is non-predictive as the salt concentration's quadratic regression give negative values of WA when S is between 10 gL^{-1} and 300 gL^{-1}. Removing the square contribution of S and the negligible interactions whose $p-$ value > 0.05 (Sigmaplot 12.5®), the model is less accurate (Eq. (2), R^2 of 62 %) but more predictive (standard error of the estimate: 10 mN m^{-1}, Fig. 5).

$$WA = \mu + \beta_1 F + \beta_2 S + \beta_3 M * F + \beta_4 M * S + \beta_5 F * S + \beta_6 M * F * S \quad (2)$$

4. Discussion

Karl-Fischer error analysis demonstrates that there is no significant difference between top and bottom samples from the same experiment (Table 2), indicating that the Frequencer™ homogenized the system in term of water content in all samples. The statistical analysis on CA results shows that the Frequencer™ has different effects on S1 solution (1 % by weight of mucin) and on S2 solution (4 % by weight of mucin) at fixed frequencies and amplitudes (Table 3). In other words, at 20 Hz and 40 Hz the pressure delivered is not powerful enough to homogenise

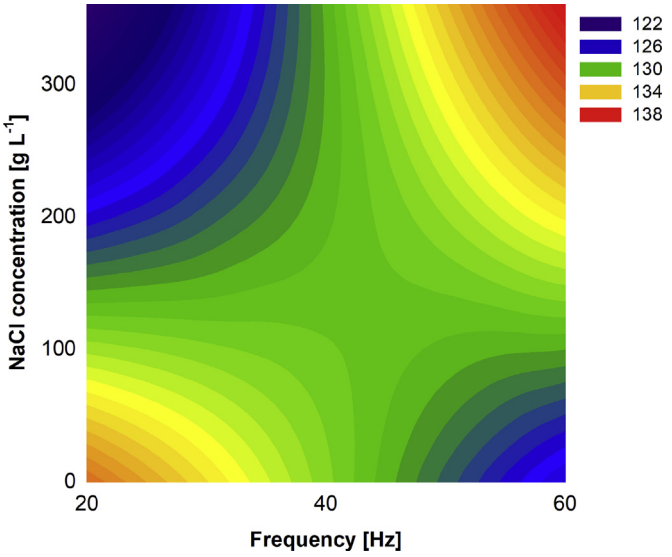

Fig. 5. WA (mN m^{-1}) response surface when the mucin fraction reaches 4 %. A saddle contour identifies the areas where WA is minimum (cold colours): lowest frequency with highest salt concentration and high frequency with no salt. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the T and B fractions and the mucus, with the mucus accumulating more at the bottom, thus resulting in a lower surface wettability.

We hypothesize that NaCl interacts with mucus above a certain relative concentration, which depends on how infrasound promotes NaCl transfer between the two and water, as a consequence. Indeed, for all S2 samples, the NaCl concentration was higher in the B phase, i.e. the mucin rich one, differently from S1 samples (Fig. 2), indicating that NaCl migrates preferentially to the mucus phase. For the saturated NaCl solution treated at 40 Hz (tests M39 and M40) the difference in the reparation of NaCl between the T and B phases is the highest, in particular at 50 % intensity, resulting in a significantly different wettability of T and B phases.

However, for both S1 and S2 with 0.5 gL^{-1} NaCl solution, the amount of salt titrated with $AgNO_3$ is actually higher than the amount of salt we added before each test. Namely, the concentration of NaCl determined was one to four times the amount expected (0.25 gL^{-1}). This might be due to the sulfur atom present on the cysteinic domain of mucins, which interacts with Ag^+ and biases the outcome of the titration. Indeed, the amount of NaCl of S2 samples (M37-M48) is higher than that of S1 (M13-M24) because there are 4 times more cysteinic domains that can interact with Ag^+.

Viscosity behaviour (Table 4) agrees with Lai et al. [42], who claim that changes in ionic strength can directly lead to shrinkage or swelling of mucus and, thus, significantly alter mucus viscoelasticity. In general, because of the higher content of NaCl in the B fraction as shown by conductimetric titrations, T samples are more viscous than B samples. In fact, an increase in ion concentration correlates with a decrease of mucus viscosity [43]. However, for tests with S2 and brine at 60 Hz and 100 % amplitude, the opposite takes place: B samples are more viscous than T ones. In fact, conductimetric titrations point out a higher concentration of NaCl in T samples than in B samples for tests M 37-M 42 (Fig. 2).

Density values confirm the sedimentation of mucus at the bottom of the reactor, as suggested by CAs statistical analysis and that the Frequencer™ imparts homogenization on the system for tests M1-M36 and M43-M48. For both T and B samples, density is constant (see Table 5).

Eventually, the work of adhesion (WA) of S1 solution, is independent from the amplitude, except at 40 Hz (Fig. 3). Moreover, there is a minimum in WA for both S1 and S2 at a concentration of NaCl of 0.5 gL^{-1}. Mc Cutchen and Wilkins observed the same trend for the mucus lubricating ability on glass [44]. We therefore speculate that high ionic strengths lead to a saturation of the negative charges of both glass and mucin's molecular chains and that the physiological NaCl concentration may be the optimum to obtain the minimum WA *in vitro*.

Concluding, cystic fibrosis is a pathology that requires permanent treatment. Mechanical solicitation, either by conventional chest physiotherapy technique or medical devices is fundamental to help patients expectorate mucus. With an operating frequency of 20 Hz to 65 Hz, the Frequencer™ provides a gentler therapy than the traditional "clapping" method of postural drainage [26]. The Frequencer™ proved to be effective in the homogenization of synthetic mucin solution *in vitro* in 20 min. A working frequency of 40 Hz and a 0.5 gL^{-1} NaCl solution are the optimal operative parameters to obtain a partial rehydration of mucus, regardless the amplitude selected, which is consistent with what reported by patients using the device. The treatment of a 4 % by weight mucus solution (S2) with a 0.5 gL^{-1} NaCl solution at 40 Hz provided work of adhesion (WA) values 17 % and 25 % lower than those obtained for S1 (1 % by weight mucus). This means that the Frequencer™ elicits improved effectiveness when the environment is richer in mucins. The main limitation of the work lies in the use of a paraffin wax to simulate lung skin. Lung skin has an inferioir elongation (150 % to 210 % [45]) compared to that of the paraffinic film (200 % to 300 %) and its average surface tension varies from 2 mN m^{-1} to 45 mN m^{-1} [46]. Another difference is the thickness of the two substrates: the alveolar-capillary barrier thickness varies from 0.5 µm to 0.7 µm [47] whereas Parafilm™'s is 130 µm [48]. It remains to assess whether a better mixing or rather the difference between the adhesive properties of the two phases is beneficial for the expectoration. However, considering that patients report improved mucus fluidity between 37 Hz and 42 Hz, we speculate that it is to ascribe to a better mixing. *In vivo* tests may confirm this hypothesis.

Author contributions statement

D.S. drafted the manuscript. D.S., R.G., F.G. and M.S. conducted the experiments with the Frequencer™. D.S., F.G., and M.S. analysed the samples except for the rheometry. F.G. and M.R. measured viscosity and density of the samples. D.C.B. conceived the experiments. All authors analysed the data and reviewed the manuscript.

Conflicts of interest

The author(s) declare no competing interests.

Acknowledgements

The authors gratefully acknowledge the support of the Natural Sciences and Engineering Research Council of Canada (NSERC) to provide support through the Engage program. This research was undertaken, in part, thanks to funding from the Canada Research Chairs program. We are very thankful to the company Dymedso, which provided us with The Frequencer™ used for the tests, technical input and helpful discussion during the realization of the project. We acknowledge Caterina Rocca and Claudio Boffito for the graphic support.

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