An Investigation into Improving the CPAP and the Electrical Stimulation for the OSA treatment

Ali Adil

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Primary Supervisor: Professor Ahmed Al-Jumaily

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Abstract

Obstructive sleep apnea (OSA) is considered a worldwide public health problem. It is characterised by the repetitive episodes of partial (hypopnea) or complete collapse (apnea) within the upper airway during sleep of OSA patients despite their continuous breathing efforts. From a pathophysiological point of view, OSA is a multifactorial disease and the mechanism underlying OSA is not fully understood. However, it is reported that it is related to the progressive loss of lingual and pharyngeal tone in the upper airway during sleep compared to wakefulness in patients with OSA. Studies have confirmed that OSA may lead to Cardiovascular diseases, Diabetes, Morbidity and high mortality rate.

Current OSA treatment include many modalities. Some are non-invasive such as the continuous positive airway pressure (CPAP), which is considered as the primary choice to manage OSA patients. While others are invasive such as the electrical stimulation techniques. However, both treatment techniques have many drawbacks. For example, the use of CPAP may be associated with numerous side effects including upper airway congestion, and significant dryness. On the other hand, electrical stimulation is associated with inflammation due to biocompatibility issue of the electrode with human tissue.

As both of the above two modalities are widely used and expanding, this thesis is focus on quantifying the damage caused by both of them and suggest possible scenarios to overcome these damages

To do so, this is a two-fold thesis. First to quantify humidification within upper airway (UAW) during application of CPAP. The study also aims to determine the impact of applying pressure oscillation (PO) waves superimposed on CPAP on humidification parameters of the UAW. Another important aspect of this research is to improve electrical stimulation therapy as an alternative to CPAP.

To address the first part, an *ex vivo* experimental setup was developed to quantify the air humidification at different CPAP operating conditions. While the *in vivo* tests were conducted using a proper clinical trial.

Results from ex vivo studies have confirmed that at normal breathing, the reconditioning of inhaled tidal volume may lead to a fluid depletion within the depth of airway surface liquid that equal to 2.17 $\mu L/cm^2$.min. However, applying the CPAP at different pressure of (5,10 and 20 cmH_2O may affect this value, significantly. Results of ex vivo studies have confirmed that a reduction percentage within tracheal Fluid depletion can be 38.4 % up to 75.8 %. The

highest reduction percentages were associated with applying CPAP at 20 cmH_2O, and the lowest effect was associated with applying CPAP at 5 cmH_2O. Data suggest a backward relationship between CPAP and TWC values of the processed air and the Tracheal Fluid depletion By applying the Pressure oscillation at different frequencies of (5, 20 and 30) Hz, results shows a remarkable improvement within the tracheal Fluid depletion at any pressure value of CPAP. However, the highest improvement percentage of 78.8 % was achieved at applying PO at 30 Hz, in conjunction with the CPAP at 5 cmH_2O. Result suggests the efficacy and reliability of applying PO as alternative to address issues associated with the CPAP.

Results obtained from clinical trial supports findings of ex vivo studies. As per data, applying full session treatment using CPAP, saliva samples collected from participants have witnessed a drastic reduction in their salivary flow rate, which was already considered as low. Reduction percentage values were ranged from 5.81 % to 70.15 %. The highest decreasing percentages within the salivary flow rates were found with participants of BMI higher than 34.4 kg/m^2. The lowest percentages were found within participants with BMI of equal to or lower than 32.8 kg/m^2. However, a major improvement in the salivary flow rate recorded from participants after CPAP and PO treatment sessions. Improvement percentages are in the range of 0.49 to 1.39 mL/min, representing an increasing percentage between 9.4 % up to 129 %. The highest improvement was recorded within the salivary flow rate of the participants with a BMI of 29.9 kg/m^2, whereas the lowest was obtained with participants of 49.1 kg/m^2. Results may confirm the efficacy of applying the PO in conjunction with the CPAP to improve dryness symptoms that mainly associate with the use of the CPAP.

To address the inflammation and poor performance caused by inflammation developed by the Electrical stimulation technique, a biocompatible electrode have been developed, Morphological studies have confirmed the capturing of cell's feature on the surfaces of electrode. Also, the implant was electrically-conductive, and results obtained from the test confirmed that both PPy/APS/ Kolliphor P188 nanocomposites and implants are electrically-conductive. Biocompatibility of implant surfaces was tested, and results obtained from corresponding tests have confirmed its non-toxicity. Furthermore, applying electrical stimulation to the attached cells has confirmed that a signal lower than 150 mV/mm can be tolerated by the attached cell population. Collectively, result may suggest the efficacy of our innovative technique to enhance electrode biocompatibility.

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Statement of Originality

" I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgement) nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution or higher learning"

……..(Signed)

…….(Date)

Table of Terms and Abbreviations

AHI	Apnea-Hypopnea Index
ASL	Airway surface liquid
BMI	Body Mass Index
CPAP	Continuous positive airway
CP	Conductive Polymers
DISE	Drug-Induced sedation Endoscopy
EMG	Electromyography
ES	Electrical Stimulation
HG	Hypoglossal Nerve
HGNS	Hypoglossal nerve stimulation
MCT	Mucociliary transport
OSA	Obstructive sleep apnea
PCL	Periciliary layer
PIS	Participant information sheet
PO	Pressure Oscillation
PSG	Polysomnography
PPy	Polypyrrole
PEDOT	Poly (3,4-ethylenedioxythiophene
PD	Electrical potential Difference
RDI	Respiratory Disturbance Index
REM	Rapid-eyes movement
SD	Standard Deviation
TWC	Total Water Content
TEPD	Transepithelial potential difference
UAW	Upper Airway
WF	Tracheal Fluid depletion

Chapter 1
Background

1.1 Introduction

Obstructive Sleep Apnea (OSA) is becoming a world health problem. In New Zealand, a study has been reported that between 8 to 12 % of people have diagnosed with OSA [1]. Various research has confirmed that disease prevalence is varied among ethnic groups in New Zealander, and it has been estimated that 12% of Maori men are diagnosed with OSA, whilst this percentage falls down to around 8% among non-Maori men. It has also been observed that there is variation within women, as studies estimate that 8.5% of Maori women have been diagnosed with OSA, while non-Maori women with OSA are 2.5% of the population. Collectively, these percentages are very concerning as it is considered one of the highest rates of OSA in the world.

In this Chapter a background of OSA is presented, along with the epidemiology of the disease. Metrics that are used to classify OSA severity are highlighted. In addition, risk factors that contribute to enhancing disease prevalence are discussed thoroughly. Furthermore, health consequences that are strongly linked to the disease are indicated. Finally, treatment options that are currently applied to patients with OSA (presented as pros and cons) are discussed, followed by a chapter summary.

1.2 Background

Obstructive sleep apnea (OSA) is considered a worldwide public health problem [2-4]. It is characterised by the repetitive episodes of partial (hypopnea) or complete collapse (apnea) within the upper airway during sleep of patients with OSA despite their continuous breathing effort [5-7]. From a pathophysiological point of view, OSA is a multifactorial disease and the mechanism underlying the OSA is not fully understood [8]. However, it is reported that it is related to the progressive loss of lingual and pharyngeal tone in the upper airway during sleep compared to wakefulness in patients with OSA [9, 10]. Various factors may contribute to this loss of upper airway tone, and it can be classified according to anatomical abnormalities.

1.2.1 Anatomical abnormalities within the patient's upper airway

Structural defects in the upper airways can play a major role in the occurrence of OSA. The sizes of upper airway's components have a fundamental role in developing this obstruction. For example, large sizes in the soft palate, uvula, and tonsils are associated with OSA [9, 11-17]. The mechanism by which one or more of these defects may contribute to OSA is through narrowing or blocking the respiratory passage during sleep of patients with OSA [18, 19].

1.2.2 OSA affected elements

Partial or complete collapse within the upper airway occurs mainly in the pharynx, amongst different upper airway landmarks. As shown in Figure 1(a-c), these Obstruction sites may include Laryngopharynx, Nasopharyngeal, and Oropharynx. The latter site considered the most common site for obstruction[20-22].

Obstruction sites within upper airways are not constant and may change from site to another. Therefore, patient may experience obstruction in one or more site depending on the sleep posture of patients with OSA [16, 23].

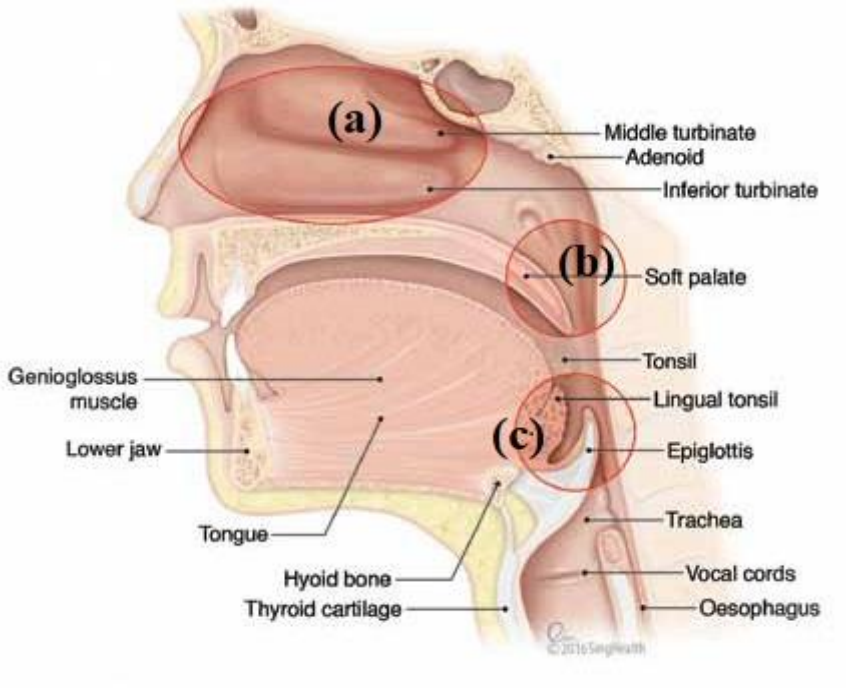


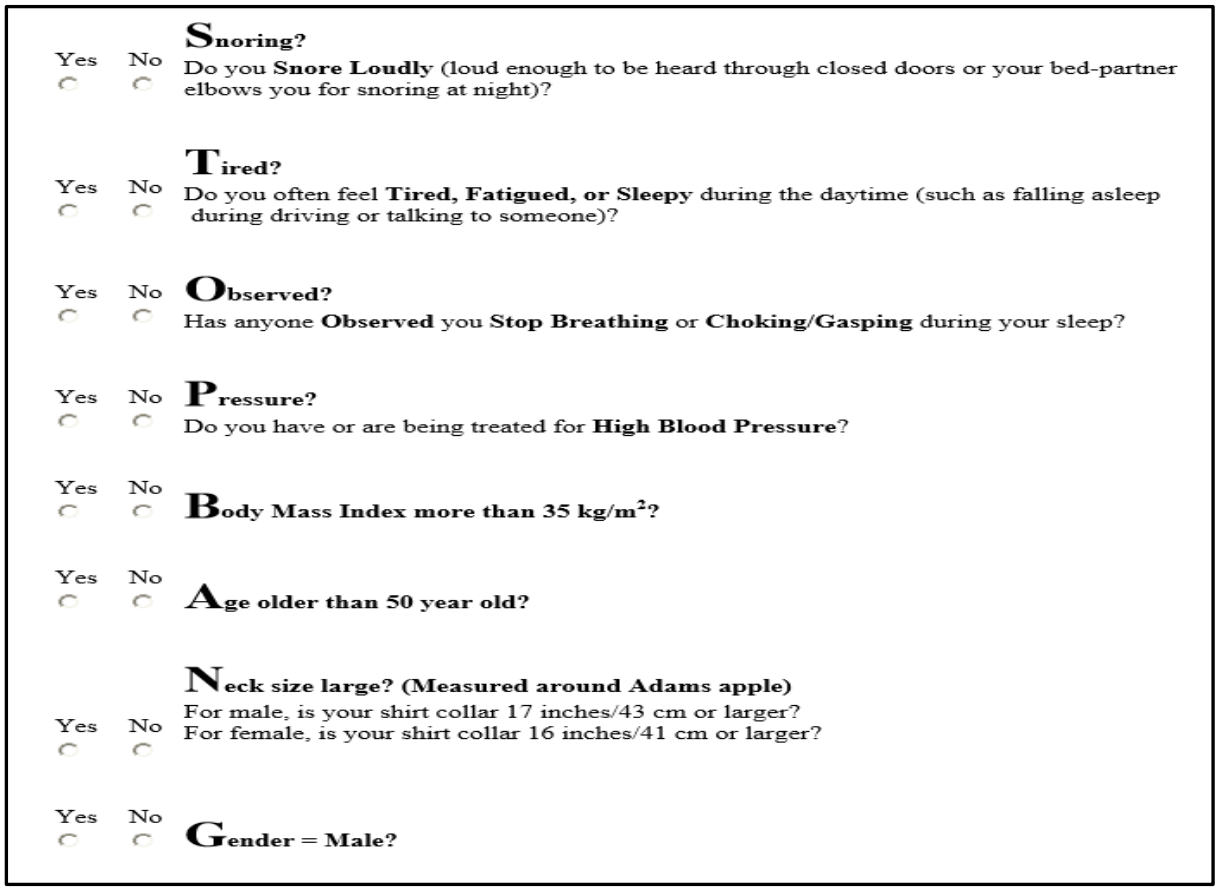
Figure 1.1: Upper airway regions that obstruction may occur; (a) refers to Nasopharyngeal affected site; (b) to Oropharynx, and (c) refer to Laryngopharynx [20].

1.2.3 Diagnosis of OSA in Adults

There are two types of diagnostic tools used to confirm whether a patient has OSA or not. These tools are paper-based and lab technique, and both are furthered discuss in the following Sections.

1.2.3.1 Questionnaire-based test

This test can be carried out through answering questions in the form of a questionnaire. Formats such as Stop-BANG, sleep apnea clinical scoring, the Berlin questionnaire, the NoSAS score, as well as multivariable apnea prediction instrument and Epworth sleepiness scale questionnaires are popular for this purpose [24-29]. The Stop-BANG test is preferable based on its feasibility and accuracy in detecting OSA. People may be considered as having a high risk of OSA when their score is equal to or more than 3. These forms are considered to be primary tests, as individuals can use them without the need of going to medical centers.



Snoring?

Yes ○ No ○ Do you **Snore Loudly** (loud enough to be heard through closed doors or your bed-partner elbows you for snoring at night)?

Tired?

Yes ○ No ○ Do you often feel **Tired, Fatigued, or Sleepy** during the daytime (such as falling asleep during driving or talking to someone)?

Observed?

Yes ○ No ○ Has anyone **Observed** you **Stop Breathing** or **Choking/Gasping** during your sleep?

Pressure?

Yes ○ No ○ Do you have or are being treated for **High Blood Pressure**?

Yes ○ No ○ **Body Mass Index more than 35 kg/m^2?**

Yes ○ No ○ **Age older than 50 year old?**

Neck size large? (Measured around Adams apple)

Yes ○ No ○ For male, is your shirt collar 17 inches/43 cm or larger?
For female, is your shirt collar 16 inches/41 cm or larger?

Yes ○ No ○ **Gender = Male?**

Figure 1.2: Form of the Stop-BANG questionnaire used for primary diagnosis tool of OSA [24].

1.2.3.2 Polysomnography (PSG)

This kind of test is the gold-standard diagnostic test for OSA [30, 31]. The test records various physiologic signals (electromyogram, electrooculogram, and electroencephalogram) during the subject's sleep. Altogether, these signals can be utilised to diagnose OSA (Figure 1.3) There are four levels of sleep testing that can be performed using PSG, depending on the recorded signals and the venue of testing. However, overnight PSG within a sleeping lab is considered the most reliable diagnostic tool [32].

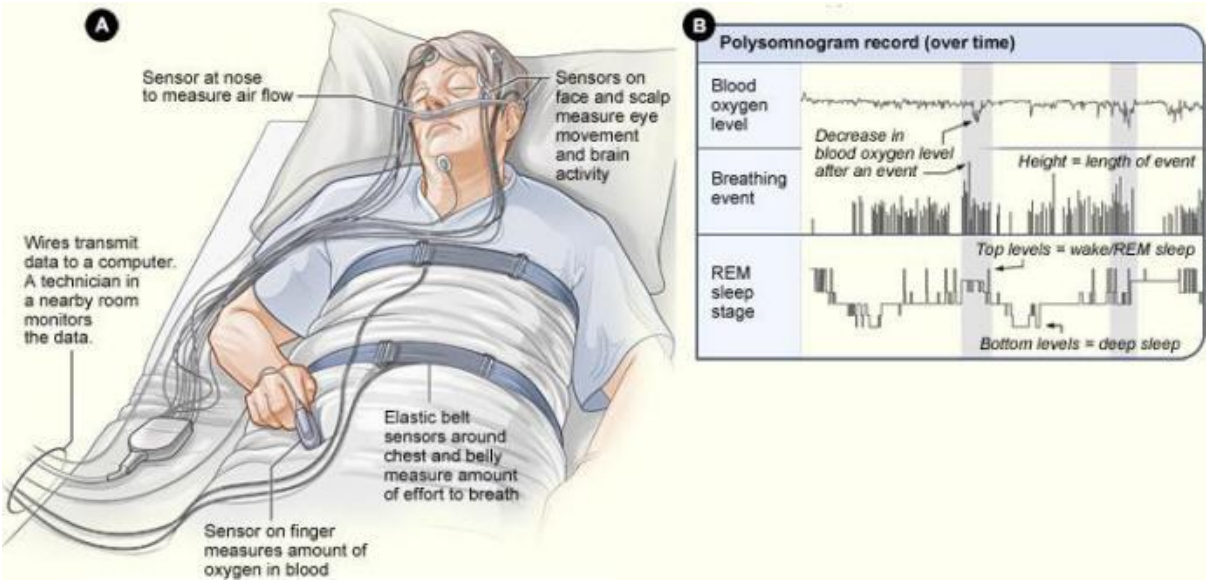


Figure 1.3: Polysomnography technique that utilised for Obstructive sleep apnea diagnosis [31]

1.2.4 Severity of the OSA

Determination of the Severity of OSA is an important tool to identify suitable treatments that need to be applied. There are two different indices used to quantify the severity of OSA, and are presented as follows:

1.2.4.1 The Apnea-Hypoapnea index

Apnea can be defined as a complete stoppage of the air flow that lasts for 10 seconds or more. Whilst, Hypoapnea is defined as a reduction affecting the air flow rate and causing arousal from sleep. The Apnea Hypoapnea Index is considered to be the standard index that can be used to define the severity of OSA, using a combined average number of apnea and

hypopnea events that occur in one hour of sleep. The severity of OSA based on the current index was developed by the American Academy of Sleep Medicine , as shown in Table 1.1 [33].

Table 1.1 Classification of obstructive sleep apnea severity according to the Apnea-Hypopnea Index

Severity	Apnea-Hypopnea Index*
None/Minimal	<5
Mild	5-15
Moderate	16-30
Severe	>30

*Apnea-Hypopnea Index is the sum of Apnea and Hypopnea per hour of sleeping

1.2.4.2 Respiratory Disturbance Index

Another index used to determine the severity of OSA is the Respiratory Disturbance Index. Unlike previous index, this one is defined as the sum of apnea -hypopnea and respiratory effort-related arousals per hour of sleep (Table 1.2). This index has been developed to characterise airflow reduction events that don't meet apnea or hypopnea time criteria [34].

Table 1.2 Classification of OSA severity using Respiratory Disturbance Index

Severity	Respiratory Disturbance Index*
Mild	5-14
Moderate	15-29
Severe	>30

*Respiratory-Disturbance Index is the sum of both the (Apnea-Hypopnea Index) and effort-related arousals of by patients counted in hour of sleeping

1.2.5 Symptoms of the OSA

Obstructive sleep apnea has many symptoms. Some of them are major while others are classified as minor, and they vary in classifications for this reason. Classification of the symptoms is established to categorise them into four main groups.

1.2.4.3 Nocturnal symptoms

Patient with OSA may experience one or more of the following night symptoms, such as Snoring loudly [16, 35]; abrupt awakening accompanied by Choking [36]; Insomnia [37]. Also, many studies have been reported other nightly symptoms are related to OSA and it include awakening with a dry mouth or sore throat [38].Night-time sweating [39], and Observed episodes of breathing cessation during sleep [40]

1.2.4.4 Day symptoms

Another category of OSA symptoms can be experienced by patient on daytime. Various reports have confirmed that patients with OSA may suffer from one or more of the following symptoms of Fatigue and difficulties in concentration [41]; Excessive Daytime sleepiness [35, 42], and Morning Headache [43]

1.2.4.5 Others

Other symptoms of OSA have been reported that mainly affect patient mentally and physiologically such as Depression [40, 44], Experiencing mood changes [45], Irritability [46], High blood pressure [47-49] and Decreased libido [50].

1.2.6 Prevalence of OSA

Although the last three decades have witnessed a comprehensive improvement in developing diagnostic tools for OSA, the majority of patients with OSA still suffer without a diagnosis. Research indicates this percentage to be between 20-80% [51]. This poor identification of OSA may lead to the severe health consequences that will be discussed below. Many factors contribute to this lack of diagnosis, such as a patient's unawareness and poor training of primary healthcare staff. Lack of diagnosis highlights the importance of gaining appropriate

knowledge. In this study risk factors that are mainly associated with OSA are identified and discussed as follows:

1.2.6.1 Age

Developing sleep-related difficulties is very common within the elderly population. Population research shows that more than 50% of people over 65 years are diagnosed with sleep difficulties [52]. Research also shows that the prevalence of OSA with aging is significant. As per the result of one of the population studies, this prevalence was more than 5 time with people between 61-100 years when compared to people between 20-44 years [53]. Another study has reported this prevalence up to 12-fold greater within older people compared to the youngest study group [54]. Similar prevalence of OSA with age advancement findings have been reported by other research groups [55].

The mechanism by which this OSA prevalence maintains a constant occurrence with elderly populations can be explained due to gaining weight. Fat mainly deposits within the neck and hence applies pressure on the muscles that are responsible to maintain the opening of the pharyngeal air flow passage [56, 57].

Confirmed prevalence of OSA in older people, particularly between the ages of 61-100 years, raises a question about whether this prevalence can lead to a high rate of mortality within this population. Research has not been able to confirm that the high rate of mortality and morbidity, the poor quality of life, attribute to this prevalence, suggesting that other factors may contribute to these rates.

1.2.6.2 Obesity

Another important factor that is classified as having a strong link to the occurrence of OSA is obesity. The advancement in lifestyle and technology have all contributed to a major change within human dietary habits, contributing to a significant increase in the population that are classified as overweight and obese. Studies report this percentage at over 60% of the total population in first world countries [58].

OSA clinical studies have confirmed the strong link between the OSA and obesity, describing it as the "strongest risk factor". OSA prevalence in obese individuals became evident with research reporting this percentage between 60-90% [59]. It has also been reported that an increase in Body Mass Index BMI from 25 kg/m^2 to above 30 kg/m^2 for men under 30 years

may increase this prevalence by four-fold [60]. Another population-based study has reported the forward relationship between the increase in BMI and OSA prevalence. Weight gain over time has been found to promote OSA occurrence and its severity[59].

Interestingly, research also noted the backward relationship between BMI and the severity of OSA. Reports have confirmed that losing weight can affect the severity of OSA, reducing it proportionally. This fact indicates that weight loss may provide a full cure for patients with OSA [61].

Determination of obesity plays a fundamental role in identifying obese-risk-related OSA. Measurements such as neck and waist circumference have been utilised comprehensively. These tools are preferable to others since they have a high accuracy in reflecting the peripheral distribution of the fat, compared to the BMI which can only be used to reflect the central distribution of fat.

The mechanism by which obesity is attributed to the occurrence of OSA is thought to be through various factors. It may occur through deposition of fat on neck circumference region can develop a narrowing within the respiratory air passage. Another scenario is through affecting muscle compensation reaction triggers by the neural system to maintain respiratory air passage. Or in some instance it may happen through altering control of the respiratory system, and /or by destabilising the tracheal caudal traction [61].

1.2.6.3 Gender

Epidemiological studies were conducted to find OSA prevalence with gender. The studies have found that men have a higher prevalence than women. Research shows this ratio between 2 to 3:1 [62]. An additional study found that men with OSA, experienced high OSA severity with higher AHI in non-rapid eye movement compared to women. Furthermore, men generally experienced longer durations of breathing pause events [63]. Differences in gender also extended to cover OSA symptoms, as studies reported that women with OSA have their own symptoms profile. For example, women are not reported to express loud snoring and gassing, characteristics that are mainly associated with OSA men [64]. However, women have reported fatigue more than men. Accordingly, reported data suggests that OSA affects men significantly.

The mechanism by which gender demonstrates distinct symptoms and distinct OSA prevalence may be explained due to different anatomical and hormonal factors. For instance, it became evident that men have a more active dilator muscle in the upper airway; have more prevalence of fat deposition in neck circumference; have a larger size of soft tissues of the UAW; and women have a higher OSA prevalence in post-menopausal than premenopausal [65, 66].

1.2.6.4 Ethnicity

Due to a wide range of clinical studies, investigating OSA prevalence in specific ethnicities is now possible. By comparing outcomes of clinical studies held in North America, Europe, and Asia regarding disease prevalence, results have confirmed that for certain ages, BMI, and sex, the severity of OSA was greater in Asian than white individuals[67] . The same observation has been noted with African Americans at ages of more than 65 years old, with a higher prevalence than other ethnic groups studied [68]. Variation of symptoms was also observed and confirmed with OSA, for example snoring prevalence has been found to be higher in Hispanic than whites[69].

Interpretation of this disease prevalence in ethnicity is challenging as different parameters may contribute to these observations. For example, the affected ethnicity is often associated with poor socioeconomic conditions. Also, it may associate with the dietary habits of the ethnic group, and lack of healthcare access.

Collectively, these parameters may contribute to disease prevalence in certain ethnic groups and regional minorities.

1.2.6.5 Craniofacial structure.

Different techniques were utilised to investigate OSA prevalence, such as radiography, tomography, and magnetic resonance imaging. Results have confirmed differences within upper airway that may contribute to the occurrence of OSA by individuals[13]. Some of the confirmed features are thought to develop OSA enlargement within the sizes of the tongue, soft palate, and tonsils [17]. Also, other structural deviations may have the same impact such as mandibular position, maxillary position, and hyoid bone position [18].

1.2.6.6 Genetics

Familial history is also considered as a major factor to promote OSA prevalence, and it was first suggested by Strohl [70]. Various studies have confirmed that individuals having a first-degree relative with OSA, are highly likely to develop this disease [71]. Another study has found that the degree of susceptibility in individuals is higher if they have a family with more members having this disorder [72]. Some of these genetic abnormalities may cause obesity as it was confirmed and hence individuals showing prevalence to the disease. Overall, the kinds of inherited genes within families likely promote OSA prevalence, and these genetics are not cured.

1.2.6.7 Addiction behaviour

Addiction to alcohol and smoking may contribute to the development of OSA. A study has confirmed that OSA prevalence in a current smoker is higher than those who are not smokers. Furthermore, addiction to alcohol can develop obstruction events within the upper airway in normal people [73, 74].

The mechanism by which individuals develop OSA is thought to occur because of inflammation within the upper airway. Thus, inflammation may alter the neural functions of the upper airway. Furthermore, alcoholic individuals may develop OSA through the effects of such addiction on consumer's respiratory motor.

1.2.7 Health Consequence of the OSA

Studies have investigated the consequences of OSA on patients. The variety of health outcomes associated with OSA is listed below in Table 1.3.

Table 1.3 Health sequels associated with OSA.

Study's finding	**References**
Hypercapnia and Hypercarbia	[47, 75]
Cardiovascular disease	[76]
Stroke	[77]
Arrhythmias	[78]
Desaturation of blood oxygen	[47]
Morbidity	[79]
High Mortality rate	[5]
Psoriasis	[80]
Hypertension	[48, 49]
Atherosclerosis	[81]
Cerebrovascular	[82]
Excessive daytime sleeping	[42, 83]
Diabetes	[84]
Nocturnal enuresis	[85]
A high rate of a car accident	[86]
Low sex-drive	[87]

1.2.8 Current treatments of OSA

The last two decades have witnessed a major advancement in the treatments of patients with OSA. Since OSA has been classified as a multifactorial disease, its management is also multidisciplinary and varies with individuals accordingly. In this study, a revision for the current treatment options is presented as follows:

1.2.8.1 Educational and behavioural treatment module

This treatment module represents the first-line treatment. The module deals with educating patients regarding the risk factors that exacerbate the severity of OSA, such as smoking, alcohol, and bad dietary habits that lead to obesity [88].

1.2.8.2 Positional therapy

This treatment module is applied when patients are diagnosed with Postural OSA. Patients may experience this kind of OSA during sleep when in the supine posture. This kind of treatment shows no improvement when it is applied to other OSA patients. This module may also include information regarding other OSA treatment modules and the best practices for using them [89].

1.2.8.3 Oral Appliances

This treatment is developed for patients with mild to moderate severity of OSA. Since it has been introduced, oral appliances have gained a lot of attention due to simplicity and affordability [90]. The mechanism by which these devices address OSA issues is through relocation of the mandible into a forward position. Therefore, treatment is prescribed to patients with craniofacial-related OSA. Although it is safe, side effects in terms of excessive saliva, teeth pain and jaw pain have been reported[91].

1.2.8.4 Surgical Treatment

Although it is considered as an invasive option, the surgical treatment module has been implemented comprehensively. Application of this option has been limited to only cases where obstruction within the upper airway passage occurs due to craniofacial abnormalities, therefore it varies depending on the primary diagnosis, as described in Table 1.4.

Table 1.4 Types of surgery interventions applied as treatment for OSA.

Type of Surgery	**Remarks**	**References**
Uvuloplatatopharyngoplasty	Within this surgery, improving the pharyngeal passage is conducted through removing part of the tissues from the uvula pharynx, soft palate, and tonsil. Although it has an acceptable success rate in diminishing the obstruction events in patients with OSA, this surgery has a poor patients' compliance due to the severe pain and also may be extended to experiencing	[92] [93]

	some difficulties in swallowing	
Uvulo-palatal surgery	It has been developed to mitigate pain associated with the previous one	[94]
Laser surgery	It is also reported for performing Uvulo-palatal surgery. Recent studies have reported that such a technique is not safe, and not recommend.	[95]
Septoplasty	It has been developed to correct a deviated septum that is thought to be responsible for nasal obstruction. The outcome of this surgery in improving obstruction and eliminating or reducing the severity of OSA is not promising	[96]
Adenotonsillectomy	This kind of surgery is recommended for obese children with OSA	[97]
Hyoid bone suspension	A multi-level treatment for patients with hypopharyngeal obstruction apnea. Due to its complexity, it has poor patient compliance. Also, it has been reported that it is associated with swallowing difficulties	[98]
Bariatric surgery	It is recommended to lose weight, which eventually leads to improving OSA	[99]
Genioglossal surgical	A kind of surgery that can be performed to remove the hypopharyngeal obstruction in patients with OSA	[100]

1.2.8.5 Non-invasive Treatment

As it has been explained earlier, surgical intervention options of OSA treatment include various disadvantages that collectively lead to poor compliance by patients with OSA. Alternative OSA therapy modules using non-invasive techniques include the following:

1.2.8.5.1 Bilevel Positive airway pressure treatment for obstructive sleep apnea.

Rather than supplying constant air pressure during inspiratory and expiratory ventilation cycles, the bilevel positive airway pressure device allows making a separate adjustment for

inspiratory and expiratory breathing. This device has been developed due to the findings that obstruction can occur in either expiration or inspiration phases. Also, it has been reported that pressure required to prevent expiratory-related obstruction is relatively lower than those necessary to prevent the inspiratory–related obstructions [101].

Various benefits are reported from using this device such as improved patient comfort. Also, this device was accepted by patients who are intolerant to the CPAP [102]. Application of the device may also be utilised to the treatment of other respiratory diseases like obstructive pulmonary disease.

1.2.8.5.2 Automatic Positive airway pressure for OSA Therapy

Automatic positive airway pressure is considered the latest edition within the positive airway pressure –based devices. The rationale underlying its development is to reduce the values of the pressure applied by the device at night, thereby improving patient comfort that leads eventually to improved patient's adherence. The device offers continuous modulation of the supplied air pressure according to the changes recorded within the airway resistance when patients change their sleep posture [103].

The mechanism by which this device adjusts the supplied air is through detecting collapse in the upper airway, and responding by increasing air pressure. Once the status of stability within airways is confirmed, then the device reduces the pressure gradually. Therefore, there is always feedback that initiates a suitable and proper action for respiration.

1.2.8.5.3 Continuous Positive airway pressure for OSA Therapy

After its development by Sullivan and associates in 1982, the CPAP has become the primary choice for specialists to manage patients with OSA. The mechanism by which CPAP addresses obstruction occurring in the upper airway is thought to occur through supplied compressed air that acts as a mechanical stent, preventing pharyngeal collapse and maintaining open airways [104]. Others suggest that air supplied by the CPAP is prompting changes in lung volumes that eventually mediates a stabilising effect within the upper airway. Regardless of the differences in the proposed mechanism of CPAP, in order to perform efficiently, compressed air supplied by the instrument must meet two criteria. First, to be tolerated by patients, there must be an acceptable pressure value range that suits human physiological conditions (temperature and relative humidity values). Second, the supplied air has a pressure value to overcome critical closing pressure due to the obstruction within upper airway [105].

1.2.8.5.4 Advantages of Continuous Positive airway pressure for OSA Therapy

CPAP technology has been utilised in clinical-based studies extensively. To confirm its impact on OSA reported health consequences, improvements in the quality of life of patients with OSA are listed below:

1. Applying CPAP therapy has reduced risk of Cardiovascular diseases [106]
2. A randomised controlled clinical trial has confirmed that applying CPAP has reduced risk of stroke on patients with OSA [107]
3. CPAP treatment in OSA is estimated to be associated with a 5.2% improvement in the Arrhythmia Risk associated with OSA [108]
4. Reported results of the clinically based study have confirmed that applying CPAP therapy has reduced morbidity and mortality [109]
5. CPAP treatment may have a positive impact on reducing blood pressure, hypoxemia, rapid intrathoracic pressure changes, and secondary hemodynamic disturbances [110]
6. Applying CPAP regularly on patients with OSA has drastically reduced desaturation of blood oxygen associated with OSA patients of OSA, [110].
7. CPAP treatment could also play an important role in improving systolic function, hemodynamics, and subendocardial ischemia [111]
8. Applying CPAP has significantly improved vascular endothelial dysfunction, thereby reducing the inflammatory response and atherosclerosis associated with OSA patients [112]
9. Applying CPAP has improved symptoms of excessive daytime sleepiness remarkably [113]
10. CPAP therapy reduced glucose levels observed in patients with OSA [114]
11. Another study has reported that applying CPAP is considered an effective treatment for reducing nocturia associated with OSA [115]
12. Applying CPAP has improved the quality of life of patients with OSA [116]
13. OSA therapy has improved some of cognitive functions [117]
14. Another finding study is that applying the CPAP has improved the sexual quality of life for women, but not men [118].

1.2.8.5.5 Drawbacks of Continuous Positive airway pressure for OSA Therapy

Despite the enormous health advancements achieved by using CPAP as first-line treatment for patients with OSA, it is associated with variously reported drawbacks. Some of these drawbacks are listed below.

1. Build-up in ear pressure[119].
2. Poorly fitting mask [120].
3. Air leaking from the mask that leads to sleep disturbance [120].
4. Chest discomfort due to exhaling against the air stream [121].
5. Intraocular build-up pressure [122].
6. Barotrauma including pneumoencephalus and pneumaothorax.[123]
7. Facial abrasions made by the CPAP mask [124]
8. Dryness in nose and mouth[125]
9. Increasing nasal congestion events and rhinorrhoea[126]
10. The requirement to repeatedly titrate the CPAP due to changes in the applied pressure.

Collectively, these drawbacks may alter patient compliance, which may hinder the benefits of consistent CPAP therapy. Some research shows patients' poor adherence to the CPAP between 40-80 % [127].

1.2.8.6 Electrical stimulation

Electrical stimulation (ES) may represent a promising alternative technique for treatment of patient with OSA. Since the emerging of ES technology in biomedical applications, it has captured numerous attentions to exploring it in new fields.

The rationale underlying developing the electrical stimulation is based on the compelling evidence that linked the occurrence of OSA with the diminished activity of the dilator pharyngeal muscle during sleep. Mainly, the genioglossus muscle is of interest, since it represents the largest muscle, and forms the major portion of the body of the tongue, suggesting the potential to be stimulated electrically for the treatment of patients with OSA [128].

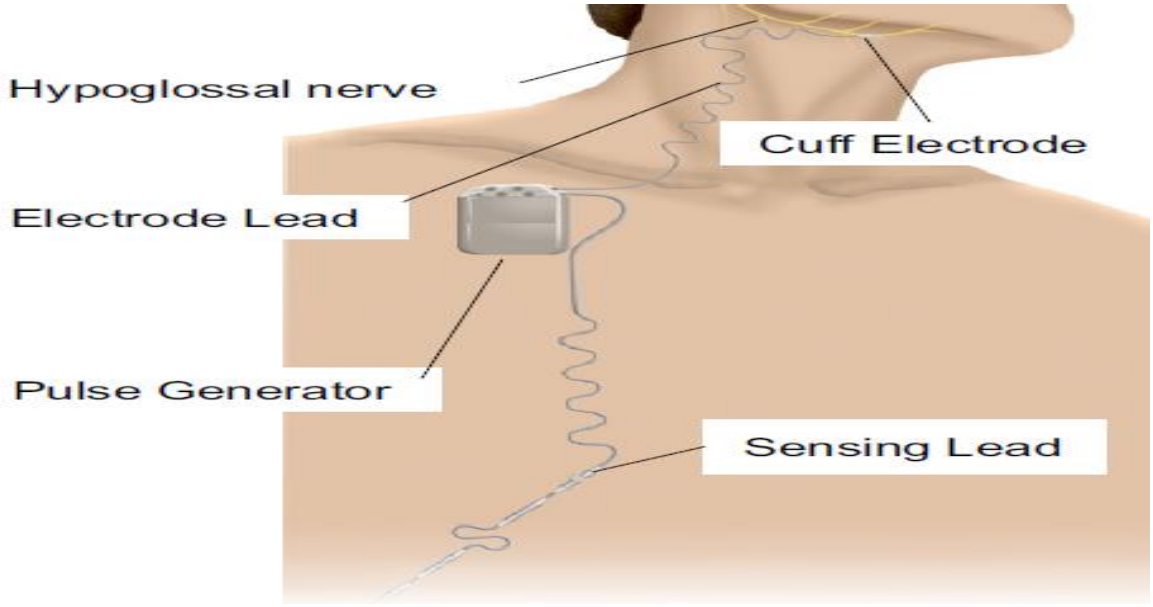


Figure 1.4; Hypoglossal nerve stimulating system components [129]

The hypoglossal nerve stimulation (HGNS) offers all the advantages of electrical stimulation, without arousal from sleep which patients experienced with the ES (Figure 1.4). The hypoglossal nerve is responsible to innervate the genioglossus muscle, and because it is classified as purely motor functioning, it is unlikely to produce arousal [130].

1.3 Closure

From the abovementioned background, the chapter highlights the main two factors which have to be considered in any OSA treatment. Factors of UAW humidification and muscle activation are thought to play a fundamental role and can dictate the success of any potential treatment of the OSA. Therefore, the next chapter will present a comprehensive literate survey of both of them.

Chapter 2
Literature Survey on upper airway characterisation and OSA

2.1 Introduction

This chapter provide a literature review on UAW humidification and what method can be used to control and improve humidification conditions for the CPAP technology. Specifically, we will focus on the use of Pressure Oscillation as a mean to improve the humidification conditions of the UAW without adding an air humidifier with the CPAP. This process requires understanding of how much the UAW can deliver and need to maintain proper air humidification condition.

The second part of this chapter focusing on the electrical stimulation (ES) and what we can do to improve the current ES technology by reducing inflammation and irritation cussed by metallic electrodes.

2.2 UAW Humification

Before entering the tracheobronchial tree, the inhaled air usually subjected to reconditioning process to be physiologically accepted by human body. These processes may include changes in temperature and relative humidity values. The UAW is mainly responsible for this reconditioning of the air through a heat transfer phenomenon between ASL and inhaled air. Consequently, process will lead to enhance air humidification condition and also to cause a fluid depletion within the Airway Surface Liquid (ASL) [131-133]. If it is not compensated, such fluid depletion will lead to health consequence as it will alter defence system and clearance mechanism by the cilia [134, 135]. Therefore, understanding and then quantifying UAW contribution that require to reconditioning the inhaled air is of a paramount importance. Below we will be reviewing previous published works.

Quantification of the Liquid secretion within UAW is investigated by Ballard et al,1999. The study was performed following *ex vivo* technique where several young pigs were sedated,

euthanised, and portions of lungs were excised and maintained in Krebs solution at room temperature. Quantification of liquid secretion was performed by aspirating all luminal liquid using 1 mL plastic syringe, followed by weighing samples using a sensitive lab scale. Research shows that the values of liquid secretion are vary from 13.4 to 19.2 $\mu L.cm^{-2}.h^{-1}$ [136]. However, this study used a small portion of bronchi, and the fluid depletion caused by reconditioning the inhaled or supplied air was not within the study scope.

The depth of tracheal ASL is thought to play fundamental role in reconditioning of the inhaled air. Maintaining the depth of ASL is important to consistently reconditioning the inhaled air. Ion transport phenomenon is thought to play a profound role to compensating any fluid depletion may affect the ASL. Research shows that trachea may respond to various chemical stimulation, differently. Such response may alter ion transport performance. Investigation to quantify ion transport performance towards various chemical stimulation was conducted by Trout et al, 1998 [137]. Quantification of the liquid secretion was performed through collecting mucus liquid using glass rod and transfer them to tared test-tube and weighed directly. The weight of collected mucus liquid was considered to be equivalent to its volume. Results come in accordance with those obtained by Ballard et al. However, this study did not provide information regarding the fluid depletion caused by reconditioning of inhaled air, and how ASL and ion transport respond toward it.

Determination of the depth of the ASL can be performed using images derived from microscopy rather than the direct collecting of liquid section. Applying this hi-tech technique is thought to provide more accurate value as it does not interfere with any mechanical stimulation. Scanning Electron Microscopy (SEM) was facilitated to quantify the depth of ASL in a portion of Bovine trachea using an innovative low temperature. Reported results have indicated that the depth of the tracheal ASL was 23 ± 6 μm [138]. Studies claimed that the obtained results came in accordance to values reported by others [139-141]. However, the study also was not able to provide a quantification of fluid depletion affects trachea during recondition the inhaled air. Also, preparation of the samples was included the immediate freezing of the samples and tested by the SEM. Such testing usually involves using of high lighting beam which may alter the actual volume of the tracheal ASL.

A developed technique using Ussing chamber has implemented to measure the depth of tracheal ASL and to measure the potential difference across epithelium of trachea isolated from guinea-pig. Measurement was conducted using KCl/agar microelectrode. Results shows

that the ASL of tracheal guinea-pig was around 200 µm [140]. Interestingly, the depth of ASL was 8-folds higher than the previous study's finding. Most importantly, fluid depletion was not investigated.

Another value for the depth of tracheal ASL has been provided, following constant-bore capillary tubes technique. Research shows that the overall liquid secretion of the tracheal ASL may reach up to 120 µL. cm-2.h-1 [142].

Reviewing aforementioned researches clearly indicated that all of them were conducted on small portions of animal trachea or lungs. Unlike others, tracheal temperature and relative humidity (RH) was investigated and measured directly using temperature and relative humidity sensors connected to thermocouples. Results have indicated that tracheal temperature was varies between 31 to 33°C, and tracheal temperature at expiration was warmer than inspiration by an average of 3 °C. Whilst, the tracheal RH has shown a constant inspiration and expiration range of 97 to 100%. [143].

Another study was performed to quantify fluid depletion of trachea that mainly caused by applying the CPAP condition[144]. Determination of tracheal Fluid depletion conducted through silica gel bag connected to cantilever scale installed within the air stream layout Experiments was designed to follow the *ex vivo* technique using a number of bovine tracheas espoused to three different pressures of 5 and 10 and 15 cm H_2O by the CPAP. The reported data suggest that:

- At zero pressure, tracheal ASL consumed around 31.2 $\mu L.cm^{-2}.h^{-1}$ for reconditioning the inhaled air.
- A reduction of 22% of the ASL and tracheal capacity has occurred due to the application of these pressure magnitudes. Surprisingly, no major differences pf applying different pressures by the CPAP were recorded in the tracheal capacity.

However, reviewing this research can highlights various gaps.

- Assumption that 0 pressure is a simulation for tidal breathing condition cannot be appropriate, since applying such conditions it has to be managed through applying Lung simulator not trapping air.

- Applying external pressure within experimental chamber as outlined in this research, is not an efficient technique to simulate pressure supplied by the CPAP, since the former is applied internal pressure.
- Researchers did not provide an evidence regarding the viability of the tissues used within these studies, particularly after 450 min. of continuous running of experiments.
- Interferences within cantilever scale due to the humidification created by the environment during process of measurement, that may lead to raise inconsistency concerns regarding the obtained results.

The effect of applying the CPAP on the UAW ability to warm and humidify inhaled air was investigated by Sahin-Yilmaz et al.,2008. The experiment has recruited 4 male and 6 female patients were exposed to trial of two various pressures of 5, and 10. Assessment of patient's ability was performed through measuring the temperature and RH of a fixed volume of cold and dry air when it inhaled and when it exhaled from nose. The reported results have confirmed the dryness associated with the using of the nasal CPAP [145].

Ulander et al.,2014 has run a prospective, longitudinal clinical trial to assess side effects associated with the long-term use of the CPAP. Study was involved the recruitment of 186 patients with OSA, 78% of them were men with median age of 58 years. Result has confirmed that 38.2 % of participants has suffering from dry mouth [146].

By reviewing the outcomes of previous researches. It is clearly indicating that none of them has given an answer regarding the amount of water consumed by tracheal ASL to reconditioning the inhaled air during normal breathing conditions, and when the elevated air flowrate supplied by the CPAP.

2.3 The activity of upper airway muscles

Understanding the mechanism underlying OSA is of paramount importance to develop alternative treatments to manage this disease. The primary UAW components that are strongly linked to the occurrence of OSA are muscle activities. Patients with OSA have a decreased activity within UAW muscle during sleep that eventually leads to promote the occurrence of either partial or complete obstruction within the respiratory passage. As

previously explained many risk factors can contribute to increasing the severity and frequency of obstruction events. Various studies report and discuss muscle activities within OSA patients, and a brief discussion of these findings is presented.

The loss in genioglossal muscle activity in patients with OSA when it compared to normal people has been confirmed, following in vivo study. Recording the activity of the genioglossal muscle and critical pressure in patients with OSA was performed using electromyography (EMG) technique. Study was performed on participants divided into two groups. The control group consisted of 12 healthy participants, with BMI of 25 kg/m^2 and no symptoms of OSA. The main group was comprised of ten males with OSA records of AHI more than 25 events per hour. The study suggests that this loss of activity may explain the frequent UAW collapse caused by a low activity of the pharyngeal dilator muscle in patients with OSA.[147].

The relationship between impairment in the dilator muscle activity and the disease occurrence has been approved by Oliven et. al.,2007. Furthermore, the study findings have confirmed that lower non-genioglossus recorded activity during sleep can contribute substantially to the poor activity of the genioglossus muscle to restore pharyngeal patency. The study suggests that monitoring respiratory patency within patients of OSA required a "high-degree" of coordination between genioglossus and other lingual muscles [148]. Evaluation of muscle activity was conducted using EMG technique by comparing muscle responses towards resistive breathing during wakefulness with their responses during sleep from patients and control groups. For this purpose, eight male patients diagnosed with severe OSA (AHI more than 45/h) and a control group of 12 healthy participants (5 men with AHI <5/h, and 7 men with AHI < 10/H) were recruited to conduct this clinical study.

Another study has reported that the genioglossus muscle response toward upper airway pressure change in patients with OSA are different when compared to those observed in the control group. The study suggests that impaired function of genioglossus muscle observed in patients with OSA may contribute to the occurrence of OSA. Study was conducted by Wijesuriya et. al.,2018 following a clinical trial with the recruitment of 13 patients with OSA and 9 participants of healthy condition to represent a control group [149]. Genioglossus Muscle response toward negative upper airway pressure was recorded using intramuscular electrodes.

Furthermore, it has been reported a consistent impaired activity of genioglossus muscle recorded by electromyography during the onset of obstruction. The study has confirmed that obstruction location in the upper airway was mainly in the oropharynx. Study was conducted by Remmers et. al.,1978 on ten obese patients with OSA, [150]. Findings of this study are inconsistent with the aforementioned studies [151-153].

Accordingly, improving the activity of genioglossus muscle and or other related lingual muscles can represent a key role to address the obstruction or collapse within respiratory passage during sleep in patients with OSA. In the following section, alternative treatment options are presented and discussed.

2.4 Pressure Oscillation waves on UAW muscles.

Various researches have been reported that applying pressure oscillation waves superimposed on CPAP can be used to as a therapy for patients with OSA.

Henke and Sullivan studied the effect of applying a high frequency of 30 Hz on upper airway muscles' activity [154]. The study was conducted on ten patients with OSA and another 6 healthy participants to acts as a control group. Supplied PO waves were performed through a nose mask. Assessment of muscles activity was performed through recording EMG signals coming from electrodes inserted in the belly of the genioglossus muscle, sternomastoid muscle, and diaphragm to assess activity during sleep. Two techniques were applied during the recording of signals, one followed rapid-eye-movement, while the other followed Non-REM. The study reported that applying PO waves on patients with OSA was found to result in notable changes.

- Applying PO waves has promoted the muscles and diaphragm activity significantly.
- Most importantly, the study reported a significant increase within the dilator muscle after applying PO waves.
- The study has suggested that such improvements with the genioglossus muscle have occurred due to the impact on upper airway receptors.

However, reviewing the study's protocol reveals that there are some limitations that may have an impact on the adaption of the study's findings. A limitation occurs in performing this investigation during the inspiratory phase of the ventilation cycle. This represents a major limitation since obstruction may occur in inspiration and expiration phases. Another

important issue is that the study did not report the value of pressure applied alongside the PO. Furthermore, the scope of this study did not include applying other medium or low frequencies. Furthermore, the study allowed the participants to sleep in any posture they prefer. There is a strong link between OSA and sleep posture during the night. Therefore, the results cannot be comparable.

The impact of applying forced oscillation at low frequency and low amplitude on the UAW muscle was examined by Badia et. al.,2001 following *in vivo* study [155]. Seven male Patients with moderate-to-serve OSA (AHI 43±21 events/h, BMI of 30±2 $kg/m^{2)}$ were recruited. Supplying PO was managed with CPAP. The activity of genioglossus muscle was assessed using EMG electrodes attached to the body of this muscle. The study was performed during REM sleep. Supplying the oscillation stimuli was not continuous, and applied only during an obstruction event and when there was a flow limitation. Reported findings of the study failed to demonstrate an increase within upper airway muscle activities including the genioglossus muscle. Therefore, no improvement with apnea-hypopnea index of patients with OSA has reported or observed.

However, reviewing the study protocol reveals a few points that may contribute to this outcome and its reported limitations. For example, patients were allowed to sleep in any sleeping posture. Also, patients were described as new users of CPAP. More importantly, patients were obese. Altogether, several factors contributed to these poor and unexpected outcomes.

the impact of applying high-frequency PO to an isolated upper airway of an unanaesthetised dog on inspiratory motor output studied by Eastwood et. al.,1999 [156]. Evaluation of the reflex effect was carried out following recording the EMG of UAW muscles. Three unanaesthetised female dogs having a weight of 20-25 kg were selected. High-frequency PO of 30 Hz and 2 to 4 cmH_2O was delivered to the dog through a mask and sublaryngeal catheters, and diaphragm activity was selected. The study found that applying PO at this condition was able to induce a tonic activation of the genioglossus muscle and to induce inhibition within the inspiratory motor output. Collectively, the applications support maintaining the UAW patency.

Another research performed by Vanderveken et. al.,2005 has quantified pharyngeal patency in a patient with OSA using forced oscillation techniques with 5 Hz frequency [157]. Eight

patients with OSA were recruited with the following criteria: their age was between 42 to 58 years, their BMI was between 24 to 30 kg/m^2, and they required RDI of 11 to 65 events/h.

The study reported that obstruction within the UAW can occur due to complete occlusion, and it can happen during the expiratory or the inspiratory phase.

Haba-Rubio et. al.,2015 have assessed a patient's tolerance to PO compared to CPAP [158]. The assessment was carried out following a clinical trial with the recruitment of fourteen patients diagnosed with OSA and current users of CPAP. Patients' characterisations were:

- Mean age was 59.9 ± 10.1 years
- BMI mean of 34.8± 7.2 kg/m^2
- AHI 58.7± 25.2 events/h

The evaluation was conducted using the Polysomnography (PSG) technique. PO waves were delivered to the UAW of patients through a mask in conjunction with CPAP at a pressure of 70% of optimal titrated pressure. Study findings have reported that there was no significant difference between the two treatment options. Reviewing this work may indicate some limitation that leads to such poor outcomes, such as

- A limited number of participants
- Relatively low BMI of patients
- The trial did not investigate the effect of this treatment on symptoms related to CPAP such as airway dryness.

2.5 Electrical stimulation techniques for OSA treatment

Various devices have been developed to stimulate the genioglossus muscle, the largest upper airway dilator muscle, to induce tongue protrusion and stiffening of the anterior pharyngeal wall [159].

The possibility of imposing a selective stimulation to the tongue's muscles, in particular, the muscles that either retract or protrude the tongue and the influence of such stimulation on the airflow dynamics during the sleep of patients with OSA, has been investigated by Schwartz et. al.,1996 [160]. Research was conducted using Fine-wire Teflon-coated steel electrostimulation electrodes (0.007 in, A-M System, Everett, WA, USA), connected to the constant-current stimulator. A trans-oral approach was selected to place the electrode to

enable stimulation of the genioglossus muscle. The study was designed to use a standard PSG technique to collect and analyse data. Nine of the Participants with an AHI of 65.5±11.5 episodes/h were clinically tested. The major finding was the capability of such a technique to selectively stimulate the specific muscle (genioglossus), and hence protrude the tongue, as the authors claimed. The tongue was observed to protrude contralaterally, upon the recruitment of the genioglossus. Results of repetitive stimulation for the genioglossus muscle showed a substantial reduction in the AHI observed, as it dropped from 65.5±11.5 to only 9.0±5.8 episodes/h. This remarkable decline is likely due to the significant improvement in the airflow patterns during stimulation, as airflow increases from a baseline value of 288.1 ± 176.2 to 501.4 ± 195 mL/s. Most importantly, collected data confirmed that such improvement was not associated with, or resulted from, the occurrence of arousal during sleep.

The effect of electrical stimulation of the tongue's protrudor and retractor muscles on the retroglossal airway wall was examined by Isono et. al.,1999 [161]. The study performed on seven male patients and an endoscopy technique was used to monitor the apparent movement of the tongue. Results came in accordance with the previous Schwartz and associates' study and confirmed the ability of selective electrical stimulation of the genioglossus muscle from restoring the patency of pharyngeal collapse. Another study confirmed that the coactivation of tongue muscles and the site of stimulation both play a fundamental role in improving the pharyngeal dimension and hence improving the airflow [148]. Study findings came in non-accordance with later studies and highlighted controversy in the method.

Due to a relatively low number of participants, the study of Schwartz et al. may not be reliable. Most importantly, the study did not investigate post-implantation safety consequences, and reports on adverse side-effects have not been highlighted. Furthermore, the long-term performance was not investigated properly. Altogether, the study raises high concerns regarding the reliability, consistency, and applicability of this technique to be adapted for the treatment of patients with OSA. Finally, these controversial findings suggest that various factors may interfere and dictate the type of tongue movement. Oliven et. al. proved that the pattern of tongue muscles recruitment may produce different tongue movement [148].

However, various studies reported the propensity of such techniques to arouse patients from sleep due to the sensory stimulation [161, 162]. Also, the inability of the genioglossus muscle to act alone to stiffening the pharyngeal from collapsing, as well as patients' discomfort and

inconsistency have been reported [129, 148]. Collectively, these limitations, makes the muscle stimulation technique less feasible and less reliable, triggering the effort to develop an alternative which can address all the aforementioned drawbacks. A technique that can offer specific targeting of the genioglossus muscle and improving patient compliance is needed.

2.6 Electrical stimulation devices

To address the propensity of arousal barriers and other negative side effects, the HGNS technique has been suggested as a reliable alternative. The HGNS offers all the advantages of electrical stimulation, without initiating arousal from sleep which patients experienced with ES.

2.6.1 Inspire I system

In one of the earliest attempts, Eisele et. al.,2000 used a direct HGNS technique to determine the motor response of the tongue muscles and to correlate these responses to the changes in airway dimensions [130]. For this purpose, five OSA patients were implanted with a tripolar half-cuff hypoglossal electrode, designed to prevent nerve entrapment. Three of the implanted patients were selected to place the electrode on the main trunk, whereas the rest selected to place the electrode on the genioglossus branch. Response to different stimulation was assessed visually, while airflow dynamics data were evaluated through standard PSG technique. Resulting data showed that stimulation of the main trunk of the hypoglossal (HG) nerve produced different tongue movements, as it causes retrusion of the tongue combined with ipsilateral deviation. Whereas stimulation of the branch of HG nerve leads to protrude the tongue with contralateral deviation. Results can be used to support the targeting of the branch of the HG nerve rather than the main trunk to achieve the preferable protrusion of the tongue. Nevertheless, both techniques were able to improve the inspiratory airflow to 184.5 ± 61.7 mL/s. Most importantly, such improvement was not associated with the arousal of patients during sleep.

The re-modulation of the stimulus intensity to maintain the device's performance, clearly indicated increasing the value of bioimpedance. Therefore, maintaining efficient long-term performance is a challenging task, with uncertain outcomes. Altogether, poor feasibility of the devices has triggered efforts to develop a new version of the device.

2.6.2 Inspire II system

In 2012, Van de Heyning et. al., 2012 performed an open perspective, pilot-scale and multicentre clinical feasibility study of the developed version of the system (Inspire II, Inspire Medical Systems, MN, USA) [163]. This study was to evaluate the preliminary efficacy and safety of the developed system in patients with moderate-to-severe OSA, those who could tolerate using CPAP. Twenty patients were selected with an AHI of 43.6 ± 8.4 episodes/h, and results of 6 months post-implantation sleep study showed a significant reduction in the OSA severity, as AHI dropped from baseline values to 7.7 ± 4.1 episodes/h. Also, the microarousal index was significantly decreased from 34 ± 12 to 9 ± 3 MAI/h. Improvement in quality of life for treated patients was evaluated using the Functional Outcomes Sleep Questionnaire form. Most importantly, the system has made a noticeable improvement in the quality of life for patients with OSA, as the results of the Functional Outcomes Sleep Questionnaire improved from 89.1 ± 23.5 to 100.8 ± 16.9. However, the number of patients who responded to stimulation of the device was poor and unoptimistic. Six out of twenty patients achieved an AHI reduction of <20 at 6-months follow up evaluation. The authors justified this poor response as a result of the higher AHI, and BMI baseline value of participants.

Although the performance and consistency in the Inspire II system reported by Van de Heyning's group was poor, a large multicentre, single group and cohort study was conducted to evaluate the clinical safety and effectiveness of this system. Strollo et al. conducted the Stimulation Therapy for Apnea Reduction, with 126 participants. Their mean age was 54.5 years, and their mean BMI was 28.4. Patients with moderate-to-severe OSA and AHI of 29.3 episodes/h were eligible to participate in this study [164].

Safiruddin et al.,2015 investigated the effect of HGNS on the retropalatal and retrolingual dimensions[165]. The study's subjects were fifteen males, with moderate-to-severe OSA and intolerance to CPAP. The subjects underwent implantation of the Inspire II system. After 2 months of implantation, both wakeful and/or drug-induced sedation endoscopy (DISE) were performed, with images taken prior to, and during the stimulation of both the retropalatal and the retrolingual regions. Endoscopy images during wakefulness and DISE showed that HGNS was able to impose an immediate enlargement of the retrolingual and the retropalatal area, and this increase was a dose-response relationship with stimulation amplitude. In DISE endoscopy where there is stimulation for sleepiness, it was noticed that HGNS produced a different response in retropalatal when compared to the retrolingual area. As the retropalatal

area was more sensitive to the HGNS and it increases by 180 %, while the retrolingual area was able to expand by 130.1% from its baseline dimensions. These differences in response were related to the shape of the affected areas as well, for instance, the HGNS of the retrolingual was more dominant and it influenced the anterior-posterior and the laterolateral dimensions.

However, the increase within the retropalatal was more noticeable and more sensitive to the HGNS when compared to the response of the retrolingual, suggesting that HGNS predominantly increased the anterior-posterior dimensions. Nevertheless, the resultant expansion was associated with significant improvements in the quality of life for patients with OSA, as it decreased the severity of AHI from its baseline value of 29 ± 3 episodes/h to 8 ± 3 episodes/h. The study suggests that this expansion can be explained by the effect of dragging the tongue's base forward resulting from HGNS. Tongue protrusion may lead to a displacement of both anterior palatal pillar and palatoglossus muscles, and hence movement of the soft palate, altogether resulting in an expansion in the retropalatal dimensions. However, the study findings are considered preliminary, suggesting more investigations for better understanding. Regarding the long-term performance of the Inspire II system, the study presented the 12 months follow up data for the treated patients. The study claimed that there were also two follow up assessments after 2- and 6-months post-implantation. No single data set regarding these visits have been reported, which raises high concerns regarding long-term performance. The study reported that patients who underwent system implantation were instructed to make follow up visits within these months, to carry out assessments using PSG and fill out a questionnaire, which eventually will be used for the system's adjustments. Various parameters were subjected to adjustment, including:

- Stimulation voltage
- Stimulation rate
- Stimulation pulse width
- Timing of electrical stimulation

Analysing the facts, the Inspire II system will need periodic adjustments, suggesting that the system was not able to achieve the same outcome in terms of reducing OSA severity over a long-term period, and therefore, the system needs re-adjustment. Manipulating the system

parameters to increase the intensity is, therefore, crucial to delivering the same relief for patients, indicating increasing the signal bioimpedance.

2.7 Drawbacks of Electrical stimulation therapy

Regardless of the type of technique, review of all previous studies clearly indicates that all devices have poor long-term performance issues, and it is likely due to the rapid increase of the signal bioimpedance, which raises very important questions in term of:

- How is the signal impedance developed over time?
- Mechanism underlying the increasing in bioimpedance
- Does the re-adjustment work effectively without boundaries?
- Most importantly what effective technique can be suggested to mitigate the risk factors related to this phenomenon?

2.8 Mechanism of developing the signal's impedance

The mechanism underlying the depression in the quality of the signal, which is intended to impose electrical stimulation for the HG nerve, is basically related to the tissue's response to the implanted electrode. Mainly, electrodes are fabricated from conductive materials such as gold, platinum, iridium oxide and glassy carbon. Besides conductivity, these materials have smooth surfaces, and this may alter the affinity and integration between the electrode's surface and surrounding tissue [166, 167]. Moreover, insertion of such metallic electrodes with hard surfaces, may trigger the immune system and develop a foreign body response; leading to developing inflammation in surrounding tissues [168]. Collectively, it will be associated with the developing fluidic gap, through which the stimulation signal has to be transduced, leading to a loss in signal quality and deficiency in the neural performance [169]. Currently, the developer of the abovementioned HGNS systems recommends periodic readjustment to overcome these problems. Readjustment of these systems can be performed by manipulating stimulation properties. Increasing the magnitude of stimulation parameters may not be suitable to maintain restoring the UAW due to exceeding the nerve excitation threshold. Also, it is reported that increasing stimulation parameters may lead to patients' discomfort and most importantly to arousals, most importantly damaging the nerve [170].

Metallic electrodes upon implantation are capable of delivering a limited charge safely. Exceeding this limit may trigger a reaction between the surrounding tissue and electrode's

surface, leading to production of gases due to changes in the pH of the microenvironment [171]. Eventually, such events can cause the tissue's death and electrode's dissolution [172].

For all the aforementioned reasons, improving the affinity and integration between implanted electrodes and surrounding tissues is fundamental for delivering high-quality signals, and to achieve consistent neural performance. It has been reported that impedance is reduced by increasing the available charge transfer area at the electrode surface, however it is recommended to impose this increase while maintaining the geometric size[173]. Furthermore, increasing the electrode's surface area could mediate an efficient transfer between biological tissue and the synthetic device [174]. Surface roughness is also known to enhance cell adhesion and tissue integration. A conductive polymer can be a promising alternative as it is considered as biocompatible, conductive, and therefore, able to eliminate the occurrence of inflammation, thereby promoting integration and maintain quality of signals.

2.9 Conductive polymers

Since first discovered in 1977, conductive polymers (CPs) sparked a lot of interest among researchers, particularly in regards to biomedical applications [175]. CPs can offer various advantages such as:

- possessing remarkable electrical and optical properties [176]
- offering the possibility to modify their electrical, chemical and physical properties (tailor-made) [177] and
- merging positive properties of the metal (conductivity) and conventional polymer (flexibility in processing).

Furthermore, CPs responding to electrochemical or electronic signals can produce a change in conductivity and volume. Based on these remarkable features, CPs have been explored in various neural engineering applications, such as neural tissue [178-180], neuronal cell signaling and recording [181, 182], neural interfaces [183, 184], and neural stimulation [185, 186]. Coating neural electrodes with CPs can significantly increase the effective surface area of the electrode/tissue interface. As a result, the capacitance of the electrode site dramatically increases, creating a corresponding reduction in electrode site impedance [187].

Conductive polymer is not conductive by itself and it needs a carrying charge to become conductive. The process by which a charge can be introduced to CPs' structure is called 'doping' [188]. The list of CPs is long and includes polypyrrole (PPy), poly(3,4-ethylenedioxythiopene) (PEDOT), and polyaniline.

The mechanism underlying the electrical conductivity of CPs arises from the presence of conjugated double bond along its backbone. Through applying an electrical potential, the introduced charge (dopant) will neutralise and disrupt the stable double bond on the backbone allowing the charge to pass through the polymer film [189].

Coating the electrode surface with CPs may produce numerous advantages, including:

- better integration between the electrode and surrounding tissues, and hence improve the signal impedance.
- maintenance of the quality of delivered signals and
- increase surface area and roughness, which may enhance tissue integration and cell adhesion.

Accordingly, CPs may play a substantial rule in improving the long-term performance of the implanted electrode, and therefore reducing the severity of OSA, improving the quality of life for patients who are using HGNS systems.

Various studies have been performed in this arena. Collier et al., 2000 developed a CPs composite using PPy doped with Hyaluronic acid and investigated the tissue regeneration and the effect of applying electrical stimulation [190]. The group developed this composite using electrochemical polymerisation technique. *In vivo* characterisation was carried out by implanting the film in rats subcutaneously. Results showed that the implanted film was well-tolerated by the surrounding tissues and no major inflammation was observed. Also, the implant was able to improve the vascularisation in the area of the implant. However, the study did not investigate the implantation of electrodes and the challenges associated with coated-electrodes post-implantation, in terms of delamination, and long-term performance.

Peptides have been suggested as a dopant to develop a PPy/ peptide CPs composite. The study was performed by Cui et al.,2003 aimed to improve the biocompatibility between electrodes and surrounding tissue, relying on the ability of the peptide to minimise the chronic inflammation associated with implantation of a metallic electrode [191]. Eventually,

it may enhance cell adhesion and therefore stabilise the long-term functionality, as the authors claimed. Development of the (PPy/peptide) CPs composite was conducted using an electrochemical deposition technique. Following deposition, the developed CPs film was implanted in six adult guinea pigs' brains.

Characterisation of the post-implantation effects demonstrated that such modification was efficient in establishing a strong connection between the implant and neuronal structure and resulting in a noticeable improvement in the signal recording. However, the study reported that the implant was not able to produce efficient long-term performance. These poor performances may occur by delamination of the composite layer from the electrode probe's surface. It was also noticed that this study has only conducted chemical stability characterisation and overlooked other characterisations such as of cyclic voltammetry and *in vivo* cell adhesion assays.

A layer-by-layer deposition was explored by He et al. to develop CPs composites using polycations (gelatin or laminin) and multiple layers of polyethyleneimine or chitosan [192]. The study was designed to improve the biocompatibility between the electrode surface and surrounding tissue, using cortical neurons for evaluation. *In vitro* test results showed Laminin -PEI provides better neuron bio-affinity and it was a neuro-integrative composite.

Biocompatibility of CPs composite of PPy/PS was confirmed with the study of George et al.,2005 using rat for the animal study. The developed CPs film was implanted into the rats' cerebral cortex [193]. The *in vitro* test confirmed the positive neuronal interaction between CPs film and cortical cells. Moreover, images of immunofluorescence revealed the tendency of the surrounding cortex to integrate the implant, demonstrating remarkable affinity between neural tissue and the developed PPy conductive polymer film.

Using Poly(3,4-ethylenedioxythiophene) (PEDOT) composite, Abidian et al.,2009 developed novel nanotubes using an electrochemical deposition to coat the gold electrode, which is designed for neural interface [181]. Characterisation of the developed neural electrode was performed using electrochemical impedance spectroscopy and cyclic voltammetry techniques to explore the conductivity over a frequency range 1-105 Hz and charge capacity density, respectively. Results showed a significant reduction in impedance as it decreased from 783.3 ± 15 kΩ to 10.7 ± 2 kΩ. Remarkably, coating the electrode with PEDOT was able to improve its CDD from 1.28 ± 0.6 to 112.4 ± 9.1 mC cm^{-2}. However, the study did not support these promising results with *in vivo* and stability studies, rising high concerns about the electrode's

long-term performance, in particular *in vivo* performance. In terms of electrode performance, results from *in vitro* characterisations cannot detail the state of the surrounding tissue/electrode interface, whether this coating will improve cell proliferation, or inhibit the occurrence of inflammation. Altogether, findings suggest the necessity of performing *in vivo* studies to investigate the reaction of surrounded tissue to the implanted electrode.

Richardson et al.,2009 investigated the biological effect of coating a cochlear electrode (four-ring platinum array) with Polypyrrole doped with para-toluene sulfonate [194]. The modified electrodes were subjected to various tests for characterisations such as electrical impedance and most importantly, *in vivo* performance. The later test was performed using guinea pigs to investigate auditory response, electrical stimulation, and tissue reaction towards implantation. The authors covered aspects which were overlooked by previous studies. Results showed significant decreases in coated electrode impedance when compared with uncoated electrodes. Post-implantation results showed that the coating of the cochlear electrode was able to decrease the average threshold shift in click-evoked auditory brainstorm response. Pathologically, the coated electrode was able to improve cellular proliferation by 10% in the surrounding tissue/electrode interface. However, the authors presented data that was collected less than 2 weeks after electrode implantation, which can raise concerns regarding long-term performance. It may take more than 2 weeks to develop significant inflammation in the implant's vicinity. Additionally, impedance measurements were tested immediately post-implantation, not after 2 weeks. Conclusions regarding this study are that it has not given reliable evidence to support using CPs within neural interface applications.

In 2010, Venkatraman et al. coated electrode array with CPs composite using PEDOT-PSS, the deposition was performed electrochemically [195]. Within this study, the authors evaluated the electrical impedance of the implanted electrode after two weeks. Although *in vitro* measurement of coated-electrode impedance showed a significant improvement, *in vivo* measurement showed different patterns. As a result, both the coated-electrode and the bare electrode impedance demonstrated a rapid increase over the first eight days, followed by a slow reduction for the last eight days of study. This unfavourable increase in the electrode impedance magnitude is believed to be associated with the formation of glial scarring in the electrode-tissue interface vicinity. The impedance increase may also be associated with delamination of a coating layer from the electrode's surface, which seems likely to happen with PEDOT use. The poor outcome of the study highlights an important question. Does using CPs composite materials prevent the occurrence of glial scarring, through presenting a

rough surface to promote cellular proliferation [196] This study has not measured the wettability as an indicator for the surface's roughness. Most importantly, it highlights the vital need for a method to confirm the presence of the coating layer after implantation, as it might delaminate during the insertion process, or at a later time.

To investigate the effect of roughness of metallic electrodes on the strong binding between CPs and the underlying substrate, Green et al.,2012 conducted research using PEDOT-Pts and Platinum microelectrode arrays [196]. Surface roughness was improved through use of a laser micromachining technique, aimed to achieve a larger contact area, and hence avoid delamination. The roughness of the developed array was confirmed by using SEM technique. Besides using a morphological test, the developed array was tested *in vivo* using a cells derived from rat PC12 cell line to investigate the proliferation of cell on the electrode's surface.

Results showed that coating with PEDOT promoted cell growth when compared to bare Pt electrodes. It was also observed that stimulation had an inhibition impact on cell growth by approximately 40%. However, *in vivo* performance of the PEDOT-coated microelectrode array was not investigated, highlighting concerns about the reliability of this innovative work, as the presented *in vitro* study was not sufficient to evaluate the effect of glial scar formation on the long-term performance of the developed microelectrode array. Green et al.'s study evaluated the delamination associated with PEDOT coating. *In vivo*, however, the study did not include any evaluation for the delamination which may occur post-implantation. Most importantly, study of implant electrodes with high-roughness surfaces is needed to improve the strength of the coating, avoid delamination, and likely prevent formation of significant glial scarring. These concerns were also not appropriately dealt with by the author in another study [179, 197].

Reviewing the aforementioned research in the field of HGNS highlights the demand to develop a polymeric medium having the necessary strength to combat the well-known delamination effect, without affecting tissue's integrity. Development of a model for the upper airway's response to the electrical stimulation of the hypoglossal nerve will contribute positively to further optimisation within the HGNS field by giving a deep vision into the mechanism of the tongue's protrusion.

2.10 Concluding Remarks

From the abovementioned literature review, it can be concluded that both the CPAP and ES are successful techniques for OSA treatment However, both have some drawbacks. For the CPAP, no previous work has:

- Quantified how much humidification is needed for the UAW
- investigated and quantified UAW contribution to conditioning the inhaled air without the CPAP. when applying the CPAP,

For the ES, the inflammation and irritation induced by using metallic electrodes represents a serious problem. Thus, an alternative biocompatible electrode is unavoidable. In particular, non-metallic electrodes are an option. then last part of this study will develop an innovative biocompatible polymeric type electrode.

2.11 Research Objectives

This is a two-fold thesis. First to quantify humidification within UAW during application of CPAP. The study also aims to determine the impact of applying PO waves superimposed on CPAP on humidification parameters of the UAW. The second aim of this research is to improve electrical stimulation therapy as an alternative to CPAP. Therefore, the main objectives of this part of the research are:

- Following *ex-vivo* technique, study will
    1. Quantify humidification in UAW at normal breathing conditions following *ex vivo* experimental setup, through using a fresh bovine trachea.
    2. Quantify the effect of applying CPAP on humidification parameters of the UAW following *ex vivo* experimental setup
    3. Determine the effect of applying PO and CPAP on humidification parameters of the UAW, quantitatively.

- Following *in vivo* technique, study will
    4. Determine the dryness associated with applying CPAP without and with PO treatments in patients with OSA, quantitatively.
    5. Develop *ex vitro -in vivo* correlation regarding the effect of CPAP without and with PO on the dryness of the UAW.

- The second fold of this study focusing on improving the current ES technology, particularly the electrodes
    6. The effect of morphological features on cellular proliferation has not been fully covered. This study will investigate the influence of morphological features of a developed Bioimprinted conductive polymeric medium on promoting the integration between the electrode surface and biological tissue.
    7. To investigate the effect of electrical stimulation on the biocompatibility of the bio-imprinted conductive polymeric medium following *in vitro* techniques

2.12 Thesis structure

This thesis is structured in the following order of chapters to fulfil project aims (Figure 2-1):

1 Current knowledge of OSA epidemiologic and current therapies to manage the disease are presented in Chapter 1.

2 Literature review on superimposed Pressure Oscillation (PO) and Electrical stimulation techniques as alternative treatments for OSA is conducted in Chapter 2.

3 *Ex vivo* tests of investigating both the effect of applying the CPAP and PO plus CPAP on humidification of the UAW, with their results are collected and presented in Chapter 3.

4 Analysis of the raw results obtained from *ex vivo* study is discussed in Chapter 4.

5 Recruitment of patients with OSA to investigate both the effect of applying CPAP-alone and PO plus CPAP on dryness, and quantifications, is presented in Chapter 5.

6 Development of a bioimprinted conductive polymeric medium and evaluation of its biocompatibility as well as assessment of the effect of applying electrical stimulation on the biocompatibility of this implant are described and presented in Chapter 6.

7 Discussion of the results obtained from this study, investigate of the *in vivo/in vitro* correlation of the relationship between applying CPAP without and with PO on dryness, are presented alongside conclusions and future work in Chapter 7.

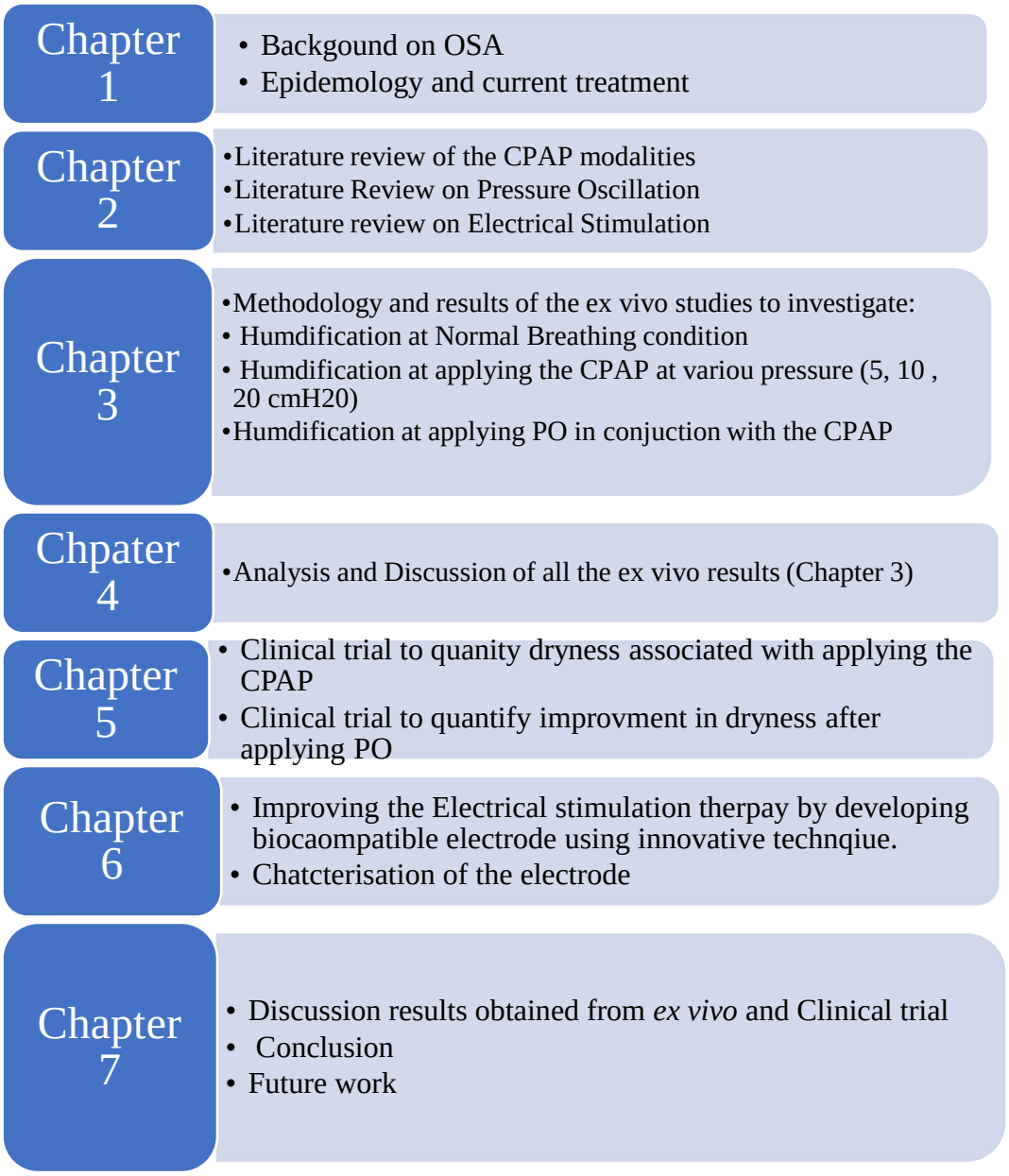


Figure 2.1 Flow chart of study' structure

2.13 Closure

From the abovementioned information, one can conclude that in any OSA treatment UAW humidification and Electrical Stimulation (ES) are to be considered as essential factors in OSA treatment. Two techniques have shown positive outcomes in OSA treatment.

One of the main drawbacks of the current CPAP technology is the UAW humidification. If no humidified air is used, dryness is a harmful condition to patients. Also, if the humidified air is applied with high water content into the UAW, it may result in discomfort and less use of the device. Therefore, it is essential to quantify how much humidification is needed for the UAW.

On the other hand, one of the main drawbacks of using the electrical stimulation is the facts that the electrodes are non-biocompatible and may lead to infection and irritation.

Thus, the following chapter will describe the methodology procedure of the *ex vivo* study to investigate the effect of applying CPAP on humidification parameters in the UAW and discuss the proposed treatment of applying PO as an alternative.

Chapter 3
Experimental *Ex-Vivo* Protocol to Quantify Upper Airway Humidification

3.1 Introduction

Various research shows information and developed mathematical modelling for upper airways following *in vivo* and *in vitro* techniques. However, the effect of applying CPAP without and with pressure oscillation (PO) waves on the upper airway has not been comprehensively investigated. Therefore, the main objectives of this study are to quantity the humidification parameters during various breathing patterns of normal breathing, and during application of CPAP at various operating pressures without and with PO waves. For this purpose, this chapter is designed to describe the experimental *ex vivo* experimental setup that is used in this study. This chapter also includes a comprehensive description for methodology, protocols and quantification parameters by which humidification of the upper airway is evaluated. Furthermore, results are obtained from the various experimental setups with their statistical analysis followed by brief analysis are presented at the end of this chapter.

Various techniques are available to conduct such investigation including *in vitro* work using cell lines, *in vivo* techniques following invasive methods, and *ex vivo* using bovine trachea. Selection criteria by which *ex vivo* is preferred is discussed in detail within the following section.

3.2 Study's Objectives

As per the introduction, the study is designed to have three aims:

(i) To quantify the tracheal capacity of re-conditioning inspired air

(ii) To investigate the effect of using CPAP on the upper airways

(iii) Investigate the effect of that using CPAP with and without PO on UAW humidification.

3.3 *Ex-vivo* general protocol

Quantifying humidity of the air within the upper airway can be performed following either *ex-vivo* or *in-vitro* techniques. The former offers more advantages over the latter, offering better simulation for the physiological environment due to the intact tissue structure, structural similarity to the human trachea, and the viability of the setup parts can be easily and efficiently maintained. Therefore, applying *ex vivo* methods promotes accuracy and consistency of the obtained results. This option can be easily followed as it does not require ethical approval.

On each day of the experiment, a fresh Kerbs solution was prepared following the formula D(+)-Glucose Monohydrate (5.55 Mm); Magnesium Sulfate (0.82 Mm); Potassium dihydrogen phosphate (1.2 Mm); Potassium chloride (3.39 Mm); Sodium chloride (110.54 Mm); Sodium hydrogen carbonate (25.68 Mm); and Calcium Chloride (2.4 Mm).The prepared solution then bubbled with (95% of Oxygen, 5% of Carbon dioxide) gas mixture for at least 10 minutes. Followed by pH adjustment to achieve pH of 7.4, and then storing them in an amber air-tight glass container at the 5^0 C condition

Figure 1 shows the experimental setup which was developed at the Institute of Biomedical Technologies (IBTec) tissue lab at AUT and it consists of a glass chamber (Figure 1 (h)) filled with fresh Krebs solution, fresh bovine trachea with a length of 28 cm, used as a model (c), glass connections designed to connect fresh Bovine trachea (e), and a temperature/relative humidity evaluation kit (f). All parts are kept in a temperature-controlled chamber to maintain the temperature at 37°C (b). In addition, this layout is connected to a lung simulator (a) placed outside the controlled chamber through a flexible hose to provide tidal breathing airflow.

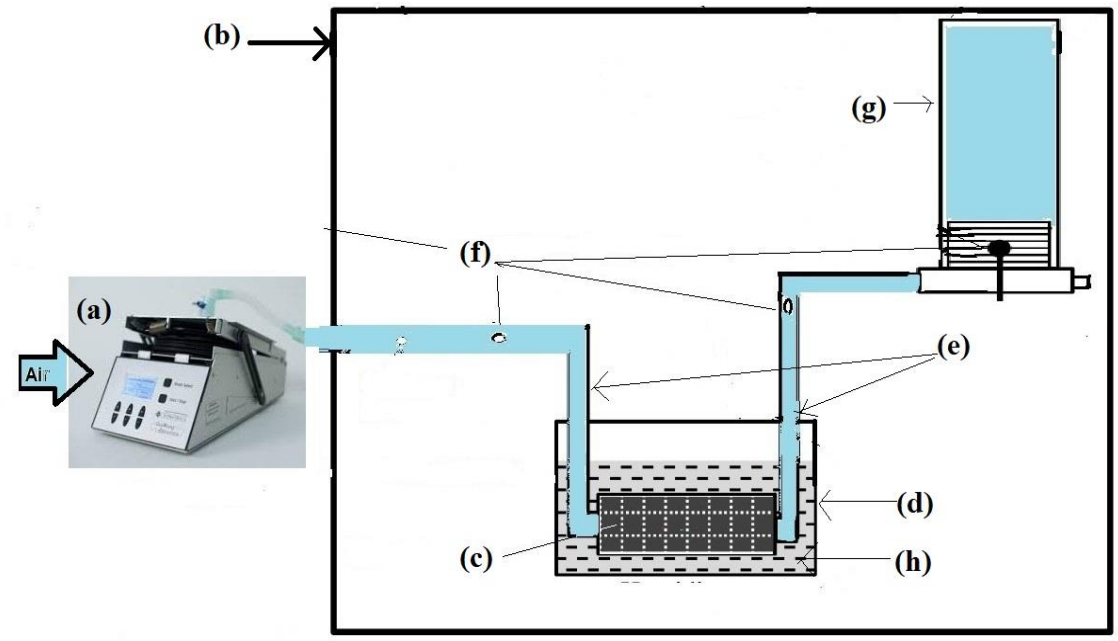


Figure 3.1. *Ex-vivo* experimental setup to simulate normal breathing conditions; (a) lung simulator; (b) modified stability chamber used to maintain temperature at 37°C; (c) bovine trachea connected to the *ex vivo* setup; ((d) glass bath used to immerse trachea ; (e) glass tube used in connection within setup; (f) temperature and Relative Humidity sensors distributed within the *ex vivo* setup to record changes; (g) plastic below used to trap air inside system; and (h) Krebs solution maintained at 37°C and used for tissue viability.

This *ex vivo* setup was designed to measure temperature and relative humidity values of the air supplied by the lung simulator (LS) at various points and over various periods of time. Quantification of the humidification in the upper airway is performed through determining the Total Water Content (TWC) of air passed through the trachea. Quantification of the tracheal Fluid depletion (WF), used to air-condition the inspired air, is also performed. Both TWC and WF values are measured based on the recorded data of temperature and relative humidity. A description for each of the equipment and experimental protocols are presented below.

3.3.1 Tracheal Preparation

Fresh bovine tracheas were obtained from Auckland Abattoir on the day of each experiment. Upon receiving the tissue, excessive connective tissue was removed, and the remainder was maintained in a polystyrene container with a sufficient amount of Krebs solution poured over it to maintain its viability. The temperature of the container was maintained at 4°C using an ice block. Prior to experimental protocols, the trachea was further processed to the desired 28

cm length required to fit within the experimental setup. The tracheal luminal surface was rinsed with a sufficient quantity of pre-oxygenated Krebs solution to remove frothy and bloodied objects. Excessive liquid was removed through repetitive tapping of the trachea.

The trachea was passed through a stainless-steel spring, developed to support the trachea within the immersion bath, and prevent any buoyancy that may occur while conducting the experiment. The two ends of the trachea were then connected securely to a modified pre-setup of the glass tube, simulating the human upper airways, using three pairs of plastic cables for this purpose. This procedure must be performed with extra caution to ensure that the whole setup is air-tight and water-tight in order to ensure the results' integrity and reliability. The whole setup was then immersed in pre-warmed Krebs solution (37°C), to simulate the physiological structure of the trachea within a human body, and to maintain tracheal viability for the duration of the experiment (Figure 3.1).

3.3.2 Temperature-controlled chamber

A stability chamber (Binder, Germany) was modified to fit the experimental conditions of this study. The whole tracheal setup and physiological bath were placed inside the modified Temperature-controlled chamber. All air connections through which LS and CPAP (ICONO series, Fisher and Paykel Healthcare) provided air flow, as well as sensor cables, were passed through a hole within the glass flap of the temperature-controlled chamber. In order to simulate physiological conditions, the temperature of the chamber was set at 37°C (Figure 3.2).

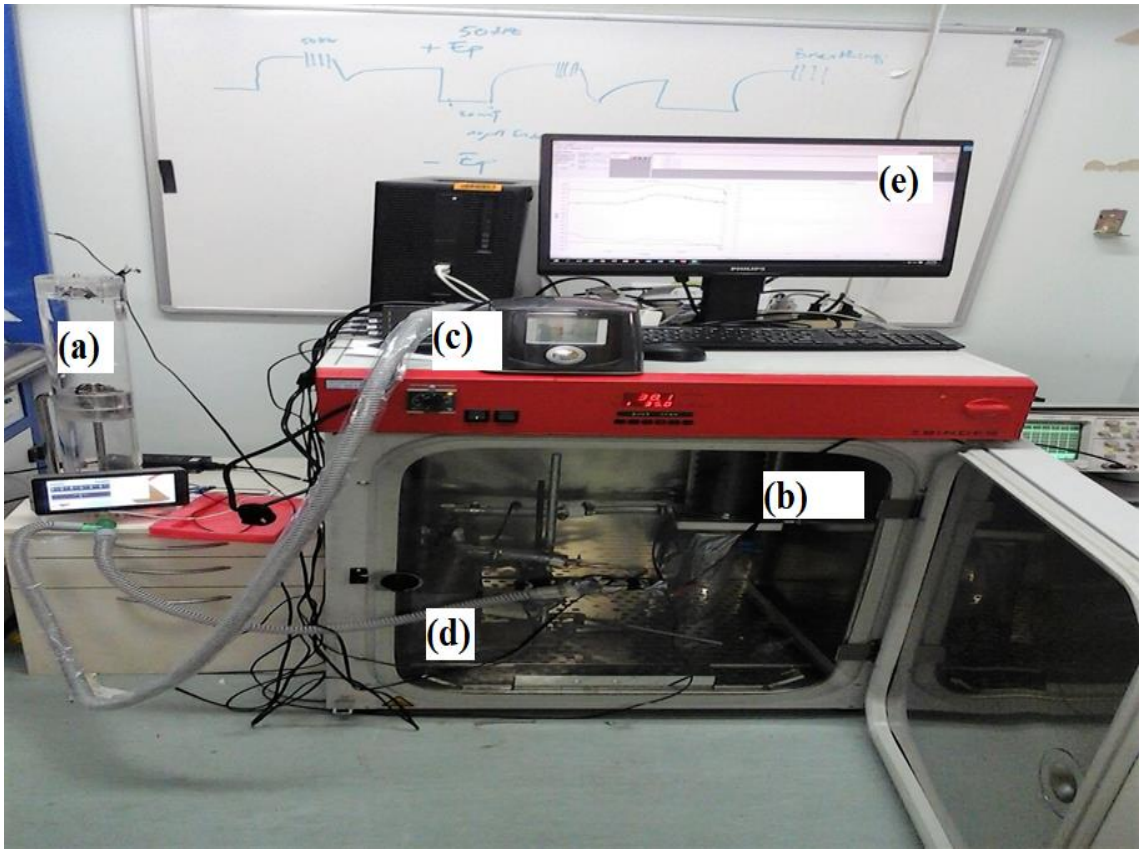


Figure 3.2. Temperature-controlled chamber layout: (a) lung simulator; (b) stability chamber maintained at 37°C; (c) CPAP; (d) tracheal setup inside the chamber, and (e) data acquisition system.

3.3.3 Lung Simulator (LS) Protocol

To simulate the quantity of air provided during normal breathing conditions, a lung simulator (F&P Healthcare, New Zealand) was attached to the respiratory simulation setup. The device consists of two parts: a mechanical portion and a control screen.

The device was developed to provide an adult air volume (500 mL) [198], and was placed outside the chamber and connected to the respiratory simulation setup through a plastic flexible hose. The device pumped in ambient quality air into the respiratory simulation setup.

3.3.4 Tracheal Viability test

All tracheas were tested prior to experimental data collection to ensure their viability. Different metrics have been reported to quantitatively characterise the mechanism of mucociliarity which can be used as an indication of the tissue viability. Methods include measuring the airway surface liquid (ASL) depth [142], the ciliary beat frequency (CBF) [199] and the velocity of mucociliary transport (MCT) [200, 201]. Among the methods, MCT

is a preferred selection for conducting the viability tissue test due to its simplicity and affordability of the required resources. A protocol was developed to perform MCT testing and an observation chamber containing a modified trough made of sylgard (Dow Corning, USA) was assembled. The tracheal dorsal muscular portion was cut along its entire length, and was incised to a set dimension (7.5 cm long and 2 cm wide). The sides of the membrane portion of the trachea were then stretched gently and pinned in the trough, as shown in Figure 3.3.

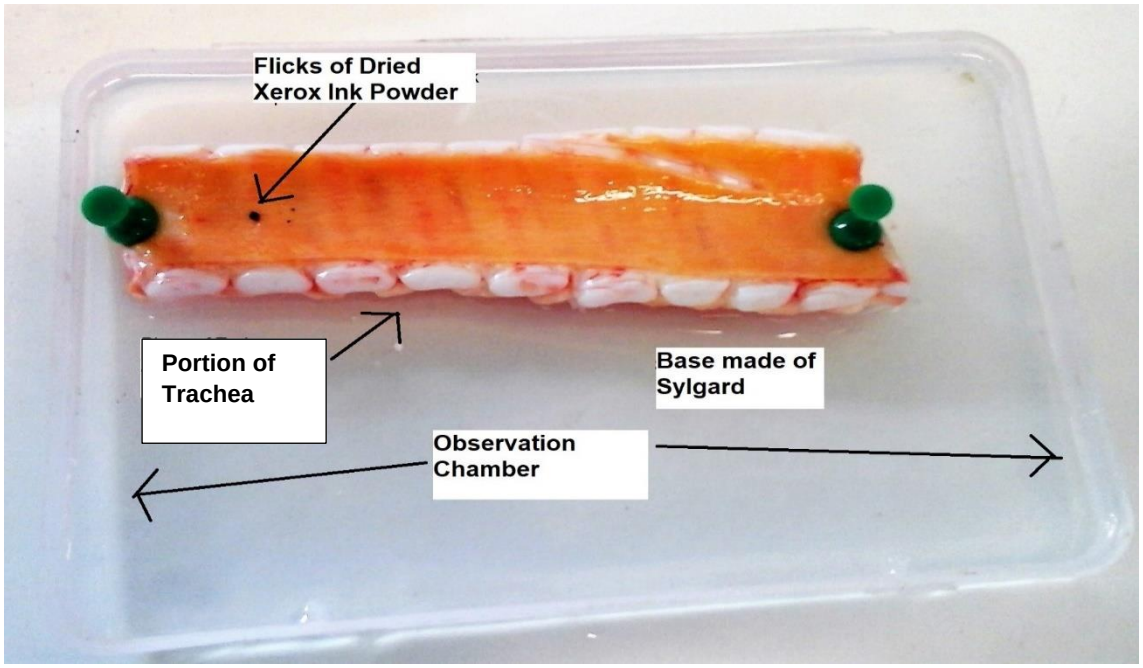


Figure 3.3 Tissue Viability test setup.

During the procedure, the basolateral surface of the trachea is submerged in a pre-warmed (37^0 C) Krebs solution. Measurement of the tracheal MCT is performed according to the following steps:

- Fuji Xerox ink powder flakes (Fuji Xerox, Japan) are applied using a micro pipette directly to the apical surface and particularly at the distal end.
- Particle movement is observed and recorded against time using a Nikon Eclipse microscope using 4x/0.10 magnification lenses.
- Data of the recorded MCT are plotted against time to build the MCT graph

Only tracheas with confirmed viability were further processed, whereas trachea failing to demonstrate viability were discarded.

3.3.5 Potential Difference measurement

Quantification of the value of transepithelial potential difference (TEPD) was performed to understand and investigate the tracheal response towards the elevated applied air pressure provided by both the LS and CPAP. In this study, the TEPD was quantified while conducting the experiment. Recording and determination of the behaviour of TEPD was performed following a protocol based on previously reported research [202, 203]. Briefly, the layout consists of two electrodes made of thin copper wire, one of them connected and attached to the apical wet surface of the trachea. The other electrode is attached to the basolateral surface, overlaying the apical surface's electrode. The basolateral electrode is isolated securely to avoid any contact with solution using parafilm (Figure 3.4).

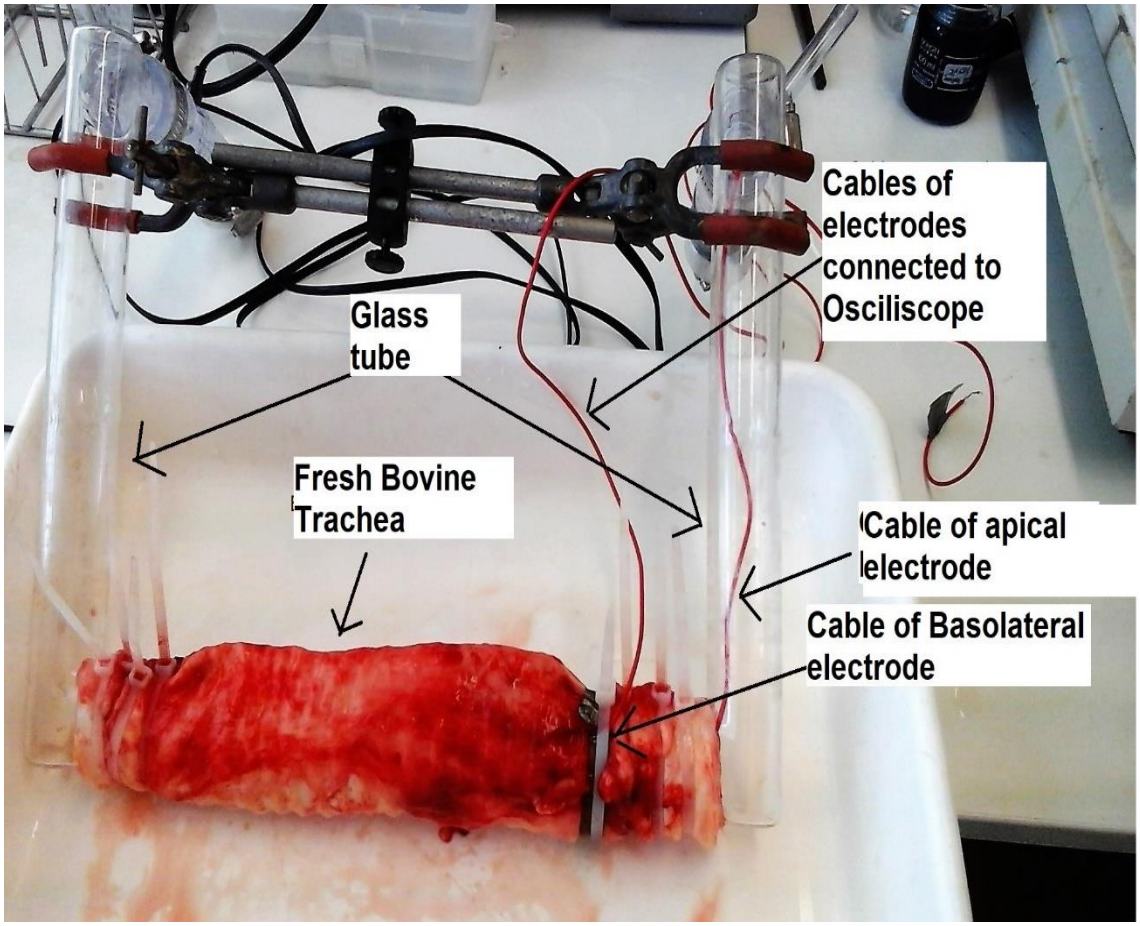


Figure 3.4 Electrodes setup to quantify transepithelial potential difference across the trachea.

Both apical and basolateral electrodes were connected to an oscilloscope (purchased from Agilent, USA), via insulated alligator cables to record the TEPD. Prior to each experiment,

the initial reading of TEPD was offset to maintain accuracy. The collected results were analysed to construct a graph through plotting recorded TEPD values verse time.

3.4 *Ex vivo* Protocol variation

The previous experimental setup discussed in Section 3.3 was further developed to investigate and meet the second and third objectives of this study. The effect of using CPAP without and with the PO was characterised following determination of the TWC of the processed air and the amount of tracheal WF parameters as discussed earlier. The modifications are discussed below.

3.4.1 Connection of the CPAP

The *ex vivo* general setup was connected to the LS and CPAP simultaneously using a "T"-shaped connector (Figure 3.2). Both devices were placed outside the temperature-controlled chamber and connected to the respiratory setup through a flexible plastic hose. The investigation was conducted to quantify alterations in tracheal thermal capacity when different operating pressures of CPAP were applied, for example, 5, 10 and 20 cmH_2O. Such influence from the CPAP on tracheal thermal capacity was quantified by determination of TWC values of processed air and the quantity of tracheal WF relative to elevated air flow rates supplied by LS and CPAP simultaneously.

For experimental consistency, a calibration setup was developed to measure the air flow rate provided by CPAP when different sets of pressure were selected using a vane anemometer (Schiltknech Messtechnik AG). Three different pressure magnitudes of the CPAP (5, 10, and 20 cmH_20) were selected; and the corresponding airflow rate was calculated based on anemometer readings (Table 3.1).

Table 3.1 Airflow rate provided by the CPAP at different operating pressure values

CPAP set pressure (cmH_2O)	The velocity of air (m/sec)	Flowrate (L/sec)
5	7.74	7.88
10	11.28	11.48
20	16.52	16.82

3.4.2 Pressure oscillation waves generator

In addition to previous investigations, the proposed improvement of PO superimposed on CPAP-supplied air was investigated following the previously described experimental setup. The setup was developed at IBTec's AUT tissue lab and it consisted of a programmable wave generator purchased from Tektronix (modelAFG3022B), connected to the pressure oscillation shaker sourced from Smartshaker (model K2004E01) 3.

Collectively, the setup generated and supplied the PO waves as a function of the input parameters of frequency and amplitude.

In this study, PO at constant amplitudes of 1 V and different frequencies of (5, 10 and 20) Hz were selected for use. Both the PO and signal generator were placed outside the temperature-controlled chamber, as explained. The PO waves were connected to the tracheal setup via a "Y"-shaped connection.

3.5 Data acquisition

The experimental setup was designed in a way to record all temperature and relative humidity readings while performing and investigating different breathing patterns. To conduct such an investigation, four different temperature/humidity sensors were sourced from Sensirion AG (Evaluation kit EK-H4, Switzerland), and were installed in specific locations within the respiratory setup (Figure 3.1 (f)). These sensors were connected to the Evaluation box through dedicated cables (cables with RJ45 plugs and pin-type connectors) in order to process data simultaneously. Visualising the obtained data of temperature and relative humidity was performed using the dedicated computer software EK-H4 Viewer (Sensirion, Switzerland).

Prior to installation, both temperature and RH sensors were calibrated following two methods. For temperature sensors, calibration was conducted against a standard thermometer, using a calibrated oven. Data were collected and analysed for accuracy. For relative humidity, sensors were calibrated against saturated salt solutions of lithium chloride (11.3%) and sodium chloride (75 %) using a humidity calibrator [204].

Finally, all data obtained from the various parts of this *ex vivo* experimental section are presented as means ± SD, with n = 7 readings. The data was analysed even further using t-test and one-way ANOVA analysis to measure significance. p-values were considered significant if the value was $P < 0.05$.

3.6 Types of measurements obtained from this study

- Temperature (°C) of inlet air, and after passing through the trachea (values obtained from the data acquisition system).
- Relative humidity of both inlet air, and air passing through trachea (values recorded using the data acquisition system).
- Data of temperature and relative humidity were processed further to calculate the TWC of processed air, as will be discussed in section 3.7.
- MCT values of the trachea (data used to indicate the viability of trachea prior to experiments).

Assessment and evaluation parameters in this work are:

- Assessment parameters under normal breathing conditions
- Assessment parameters using CPAP
- Assessment parameters under combined CPAP with PO conditions.

3.7 Results

Data collected from different points of the experimental *ex vivo* setup for air quality parameters of the supplied air were recorded in real time using Sensirion EK-H4 software. This program measures the changes in temperature and relative humidity values of the air directly from channels 1-4 of the evaluation kit equipment.

3.7.1 Data Analysis

Raw data of temperature and relative humidity of air supplied by LS and other means were processed further to determine the TWC of the treated air and tracheal WF of the trachea, quantitatively. Calculations of these two parameters were performed using the following equations to derive the values of TWC of processed air as a function of time, , parameters and numbers including within this equation are obtained from various reported researches [143, 205]:

$$\text{TWC} = \frac{\left[2.32 + (0.59\,\text{T}) - (0.011 * \text{T}^2) + (0.00063 * \text{T}^3)\right] \text{X RH}}{100 * \text{D}}. \qquad \text{(Equation. 1)}$$

Where,

D= is the density of air at 20^0 C

T= temperature of the processed air (^{0} C)

RH = relative humidity recorded by sensors

(2.32, 0.59 ,0.00063) are derived from Psychometrics chart.

The tracheal Fluid depletion (WF) values are derived from the quantification of TWC values of the air samples. Results were further analysed using Equation 2 to determine the quantity of tracheal WF.

$$\frac{dWF}{dt} = \frac{dTWC\ (post\ trachea)}{dt} - \frac{dTWC\ (Inlet\ air)}{dt}. \qquad \text{(Equation. 2)}$$

3.7.2 Assessment parameters under normal breathing conditions

Prior to the start of the experiment, the trachea are subjected to a viability test according to the protocol outlined in Section 3-4 [206].

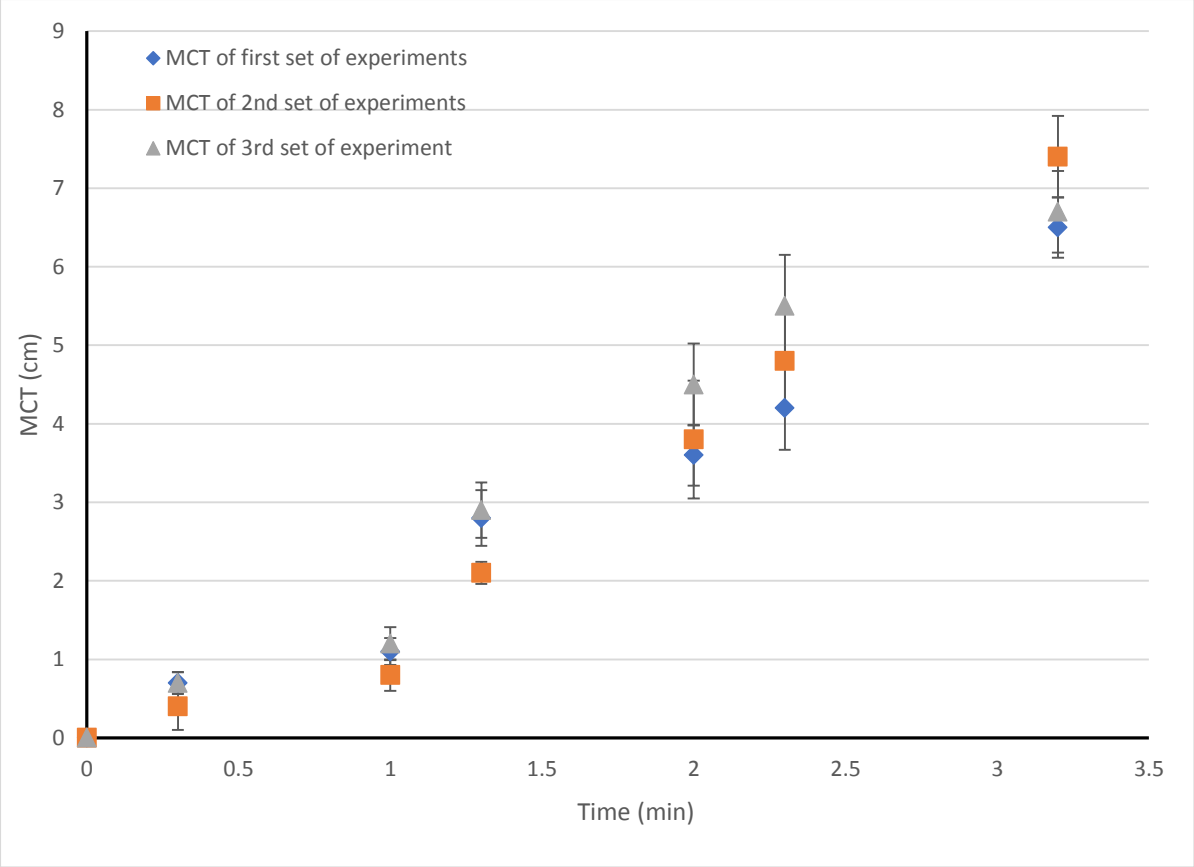


Figure 3.5 Mucociliary Transport activity of tracheas (values presented as means ± SD) for n = 7 samples.

As per Figure 3.5, the clearance distance was recorded as a function of time. and was repeated immediately following the end of each experiment.

All tested tracheas presented a forward relationship between the MCT and time with a mean MCT value ranging from 2.03 to 2.31cm.min^{-1}, confirming tracheal viability of apical tissue pushing the applied dried ink particles from the dorsal end towards the ventral end. Results obtained from *ex vivo* testing compares with the MCT ranges reported by other research [207, 208].

The results have demonstrated a heterogeneous movement pattern. Many factors can be contributed to this heterogeneity in the MCT, including but not limited to, the age and the health status of the animal. Most importantly, the size of the applied particles of the dried ink was found to have a major impact on movement. The larger flakes of ink placed on the apical surface produced slower MCT values. Consistency over the size of applied ink presented a challenge due to limitations of the apparatus used in this experiment. and required extra

cautions and practice. Consequently, the viability of the trachea was observed and recorded to support the reliability and accuracy of *ex-vivo* results.

Data suggest that all these tracheas were viable during the experiment time. The result may also indicate the effectiveness of both the tracheal handling protocols and the developed protocol of MCT quantification.

Following the confirmation of the trachea's viability, tracheas are connected to the experimental setup as described in Section 3.3 to determine the TWC of processed air and tracheal WF during normal breathing conditions. Both parameters are believed to serve as a reliable tool for humidification status of the upper airway and to quantify tracheal thermal capacity. The initial investigation determined these parameters at normal breathing conditions.

Table 3.2 Total Water Content of the processed air supplied by LS and Tracheal Fluid depletion, results presented as mean ± SD, n = 7.

Time (min)	TWC (mg/L)	WF (mL)
0	0	0
2	8.9 ± 0.6	11.9 ± 1.4
5	15.4 ± 1.1	26.6 ± 1.3
15	15.1 ± 1.8	42.9 ± 1.4
30	17.5 ± 1.8	59.7 ± 1.5
60	17.5 ± 1.3	76.6 ± 1.7
90	17.7 ± 1.7	93.7 ± 1.4

Values of TWC and tracheal WF are presented in Table 3.2. Data shows a rapid increase that lasts for 30 min., followed by a steady state phase where TWC values of the processed air were almost constant. Within the rapid phase, the trachea was able to recondition air supplied from the lung simulator efficiently, improving TWC of the air from a baseline value (ambient conditions) up to 17.1 mg/L. After 30 min., TWC values demonstrated a steady state pattern where the TWC values were slightly increased over the remaining time of the experiment, reaching 17.7 mg/L after 90 min. The result confirms that there is a relationship between accumulative tracheal WF and time with Regression value (R^2) of 0.9972.

3.7.3 Assessment parameters under the CPAP

Applying various operating pressures from the CPAP, following the described experimental setup, produced different patterns for TWC values of the air supplied by both LS and the CPAP

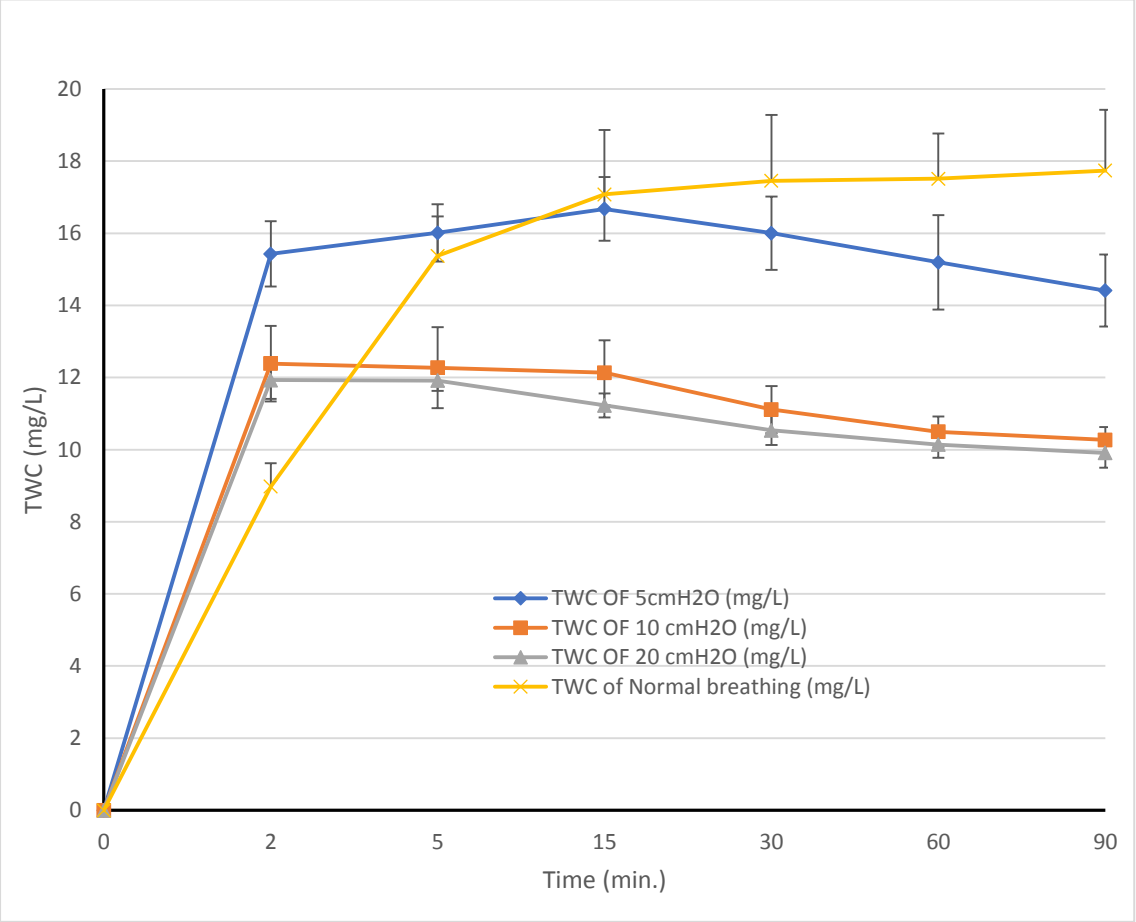


Figure 3.6. Total Water Content in the inspired air after passing through the trachea (values presented as mean ± SD, n=7).

Results of the TWC for this part of the investigation are depicted in Figure 3.6 and reveal two key observations regarding TWC values obtained at application of normal breathing conditions. First, all experiments with the different operating pressures from the CPAP have shown an identical pattern for the TWC of the delivered air, where there is a rapid increase followed by a steady phase. However, there is quite a difference in the values recorded. For example, applying lower operating pressure by CPAP improved TWC up to 17.1 mg/L after 30 min. While applying 10 and 20 cmH_2O has improved TWC of the processed air to 12.1

and 11.2 mg/L, respectively. After 90 min. these values deteriorated to 10.3 and 9.8 mg/L, respectively.

Results of accumulative tracheal WF collected from applying CPAP at a different operating pressure of (5, 10, 20 cmH_2O) were presented vs time as shown in Figure 3.7 for comparison with results obtained at applying normal breathing conditions.

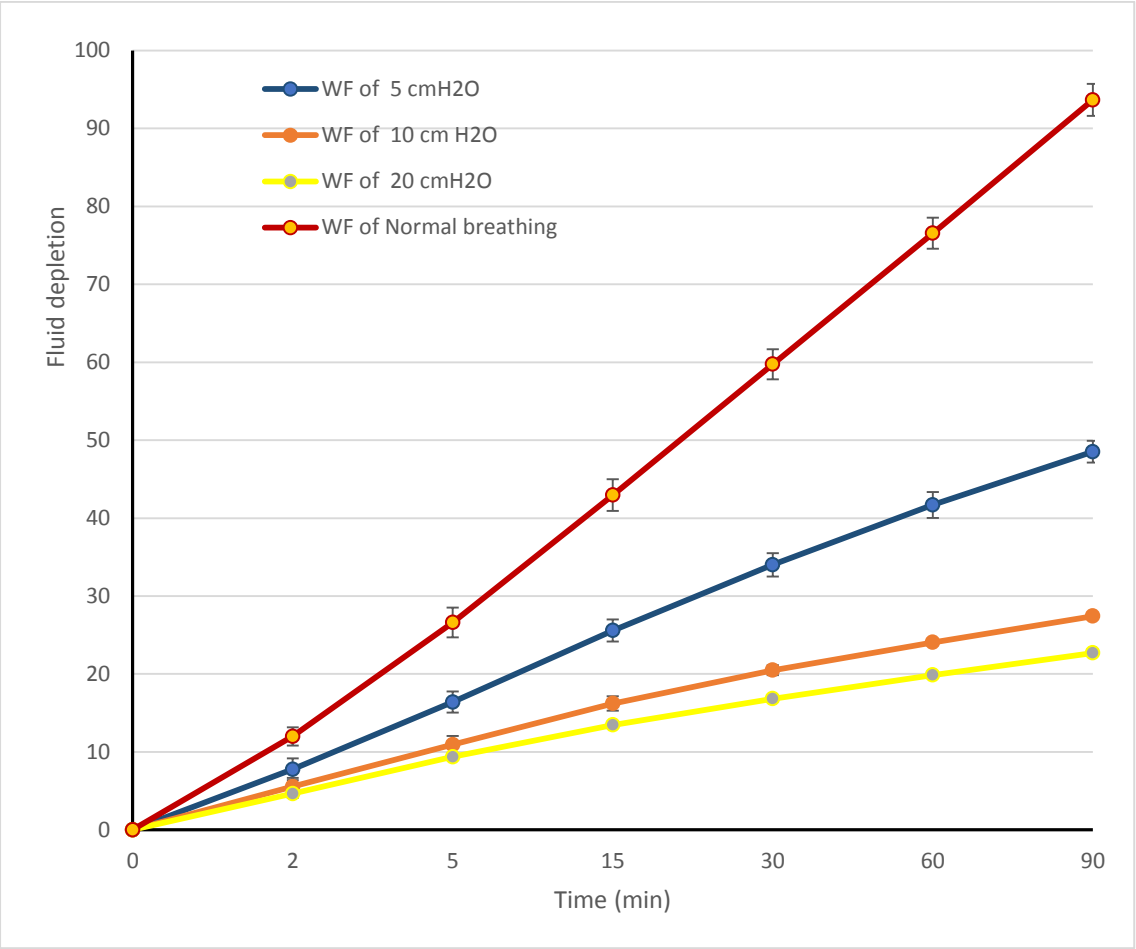


Figure 3.7. Accumulative tracheal Fluid depletion (WF) facilitated to conditioning the inspired air (presented as means ± SD, n = 7.

Overall, results show a forward relationship between accumulated tracheal WF vs time, similar to those obtained at normal breathing conditions. However, there was a drastic decline within accumulative tracheal WF values. As per results obtained from the experimental setup, applying 5 cmH_2O has decreased accumulative WF to 48.5 ± 1.7 mg in comparison to WF of 93.7 ± 2.1 achieved at normal breathing conditions. Whilst applying higher CPAP pressure at 10 and 20 cmH_2O has deteriorated accumulative tracheal WF values to low as 27.4 ± 0.2 and 22.7 ± 0.14 mg, respectively.

3.7.4 Assessment under PO

Pressure oscillation (PO) superimposed on CPAP-generated airflow has been suggested as an alternative to mitigate the restricting effect of CPAP on the tracheal thermal capacity. An investigation was conducted following the same *ex vivo* techniques as presented. Connection of the PO with CPAP is performed as explained in Section 3.4.2. In order to investigate the effect of various frequencies of PO on each level of the operating pressure of the CPAP, the study was designed as follows:

i. Effect of PO frequencies with the CPAP at 5 cmH_2O
ii. Effect of PO frequencies with the CPAP at 10 cmH_2O
iii. Effect of PO frequencies with the CPAP at 20 cmH_2O

For each investigation, an assessment was carried out by calculating the TWC of the processed air and tracheal WF.

i. Effect of PO frequencies with the CPAP at 5 cmH_2O

Regarding the quantification of TWC of the passing air, Figure 3.8, shows that applying the PO waves at the investigated frequencies (5, 20 and 30 Hz) in conjunction with the CPAP at 5 cmH_2O has promoted the TWC values after 30 min. to 20.2 ± 1.5, 22.3 ± 1.5 and 24.2 ± 1.8 mg/L respectively. At the end of experimental time, the TWC of the processed air has shown a steady state with the same recorded values.

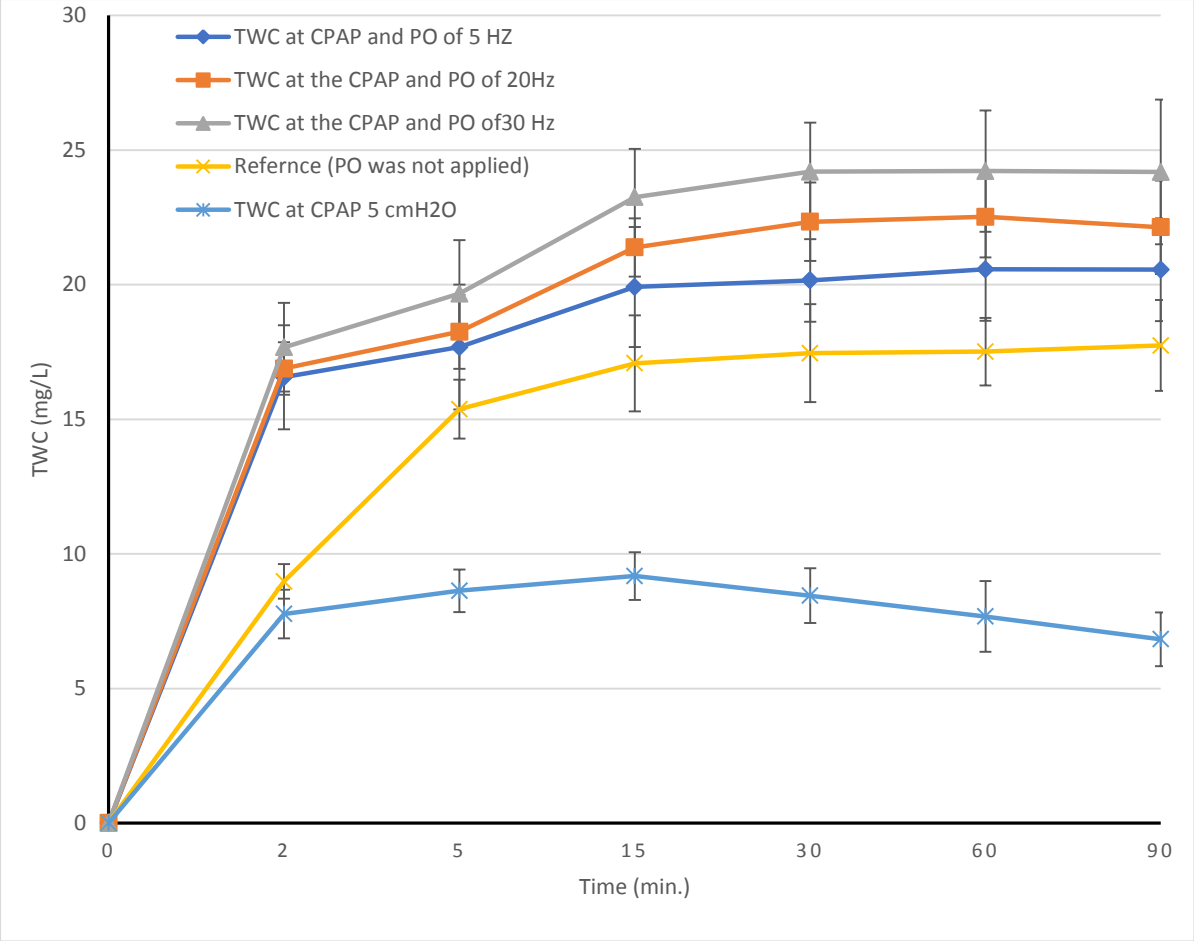


Figure 3.8. Total Water Content within the inspired air after passing through the trachea (values presented as mean ± SD, n = 7.

Regarding tracheal WF, data of TWC trials were further analysed using Equation 2 to quantify accumulated tracheal WF at different frequencies of PO. Results were then used to construct the graph in Figure 3.9.

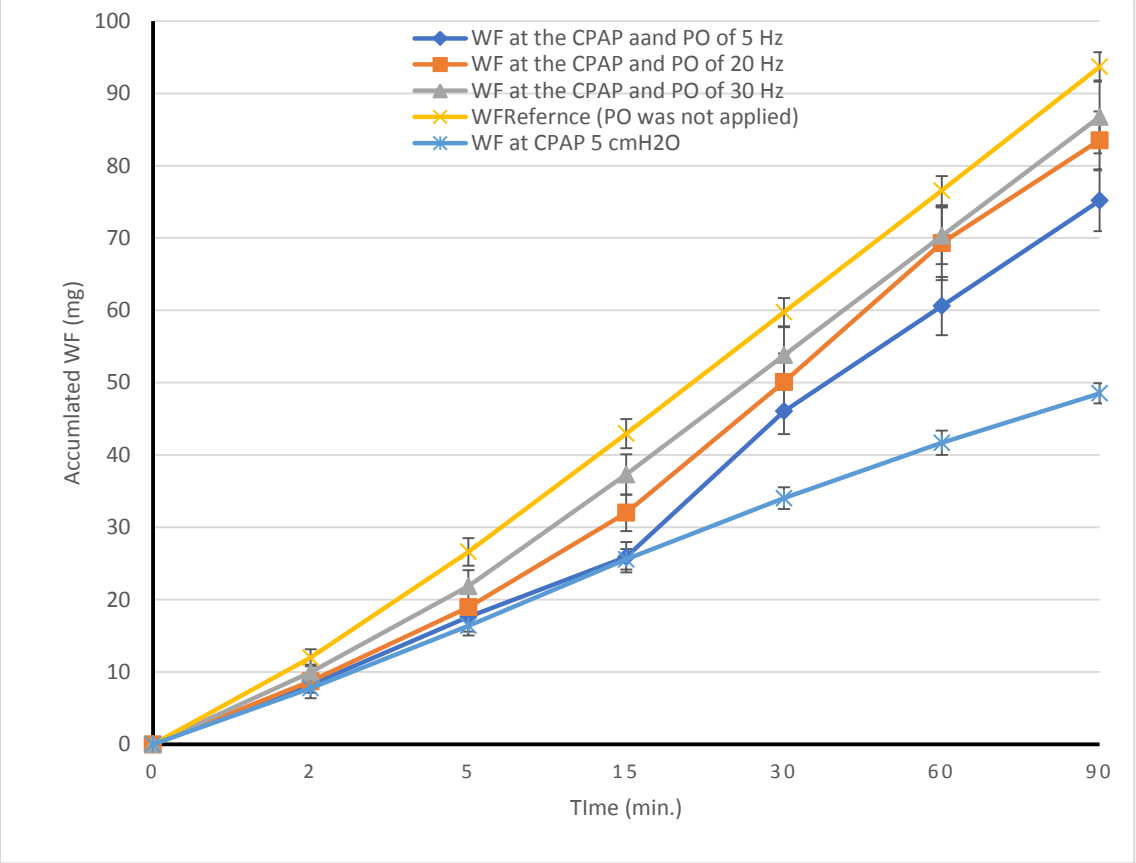


Figure 3.9. Accumulated tracheal Fluid depletion (WF) facilitated to conditioning the inspired air (values presented as means ± SD, n = 7.

Figure 3.9 shows a linear relationship between the accumulative tracheal WF and PO operating conditions. At different frequencies, PO promoted the WF. The highest improvement in accumulative WF of 86.75 ± 5 mg was achieved at 30 Hz. The applied PO at a low frequency of 5 Hz was shown to have a less efficient influence on improving the WF with a value of 75.2 ± 4.2 mg.

ii. Effect of PO frequencies with the CPAP at 10 cmH_2O

Data were collected, analysed and presented for CPAP settings of 10 cmH_2O. As shown in Figure 3.10, results of TWC have shown a rapid increase when compared to the reference status of applying only CPAP at 10 cmH_2O. Results show that applying 5, 20 and 30 Hz frequencies of PO caused a rapid increase in the TWC values of the processed air to 13.2 ± 1.5; 13.5 ± 1.6 and 13.6 ± 1.9 mg/L, respectively. However, this improvement was temporary and lasted for 5 min. of the total 90 min. The second phase was predominantly a decline in

the TWC values that continued over the remainder of the experiment. As per other results, applying different frequencies of PO (5, 20 and 30 Hz) alongside the CPAP at 10 cmH_2O, improved TWC values of the processed air up to 10.4 ± 1.4, 11.3 ± 2.1, and 12.4 ± 1.7 mg/L respectively. By comparing these values to TWC of the processed air recorded at applying only CPAP at the same pressure (10.3 ± 0.36 mg/L), the results represent a subtle impact on the TWC values of the passing air.

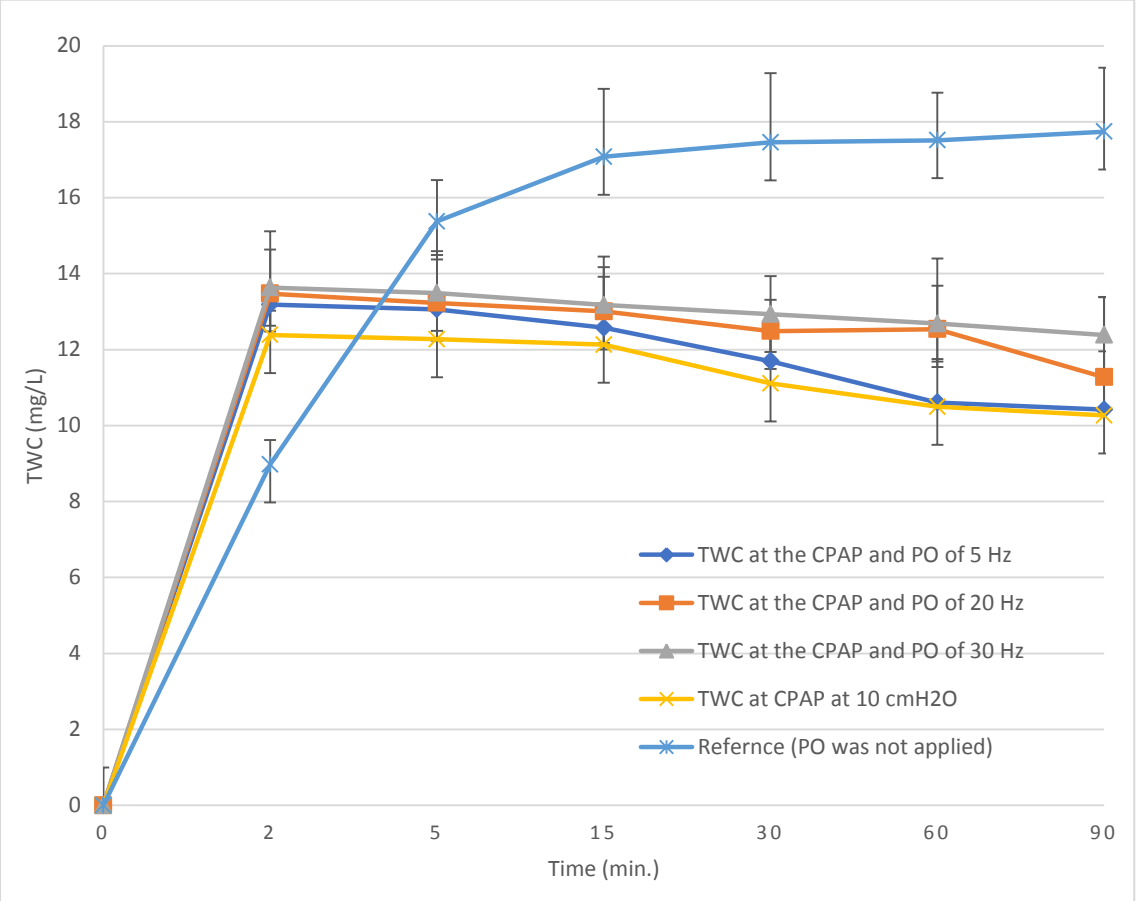


Figure 3.10. Total Water Content (TWC) of the air passing through the trachea with values presented as mean ± SD for n = 7 samples.

Analysing data for this set of experiments was conducted to calculate the tracheal WF as per Equation 2. Results depicted in Figure 3.11 have confirmed that different frequencies applied by PO have clearly promoted the WF values when compared to accumulative WF obtained by applying CPAP at 10 cmH_2O. As seen in the graph, the highest improvement is recorded at applying the higher frequency of 20 Hz, as it increases the accumulative WF up to 41.5 ± 1.7 mg compared to 27.4 ± 0.2 mg recorded at applying CPAP alone. The lowest

improvement in accumulative WF values (34.5 ± 1.3 mg) was recorded at applying PO with 5 Hz frequency.

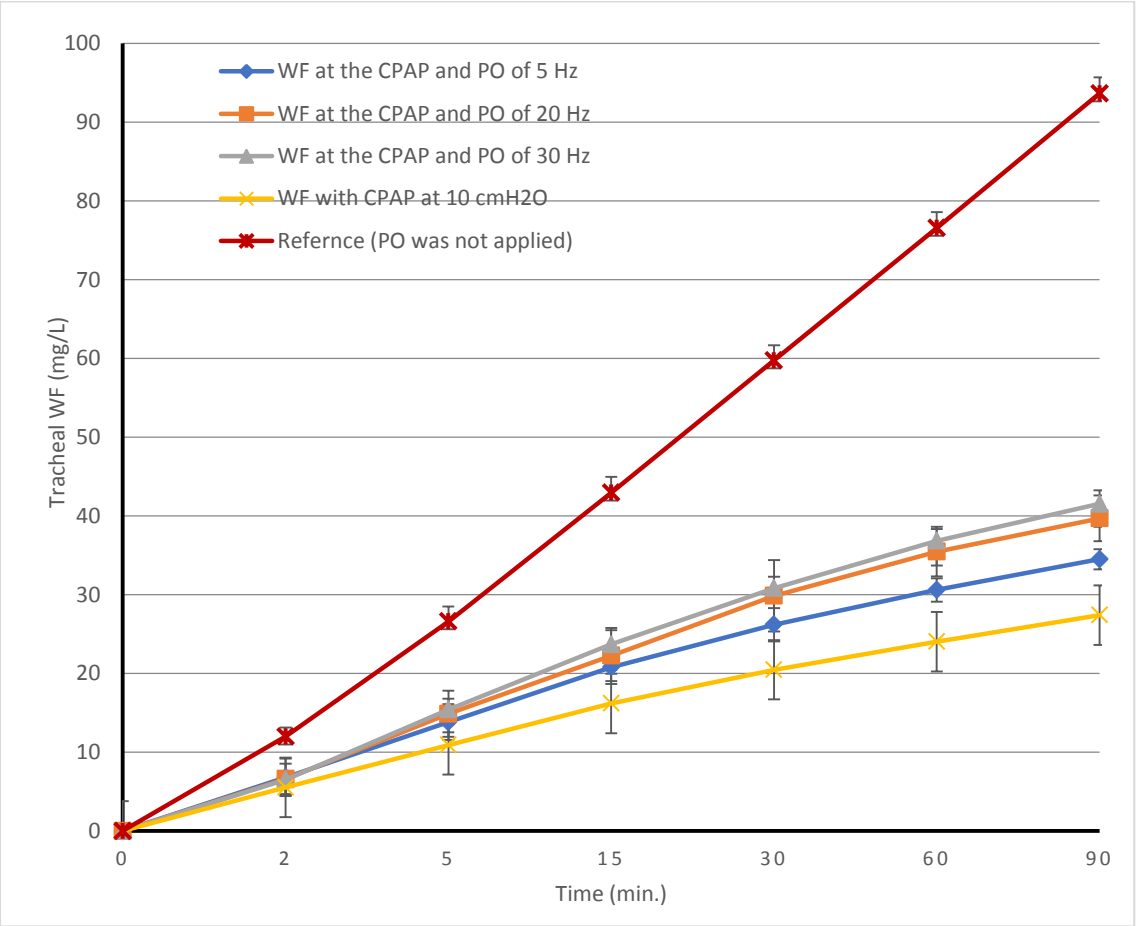


Figure 3.11 Accumulated tracheal Fluid depletion (WF), with values presented as mean ± SD for n = 7 samples.

iii. Effect of PO frequencies with the CPAP at 20 cmH_2O

Figure 3.12 shows that applying PO waves at any of the investigated frequencies has decreased the TWC of the processed air when compared to those values obtained at applying CPAP (9.9 ± 0.4mg/L). Applying 5, 20 and 30 Hz frequency by the PO in addition to the CPAP at this pressure deteriorated the TWC values drastically to 9.7 ± 0.9, 9.2 ± 1.3 and 8.7 ± 0.7 mg/L, respectively. The lowest decline is observed at applying 5 Hz, whilst the most severe reduction is recorded at applying a higher frequency of 30 Hz.

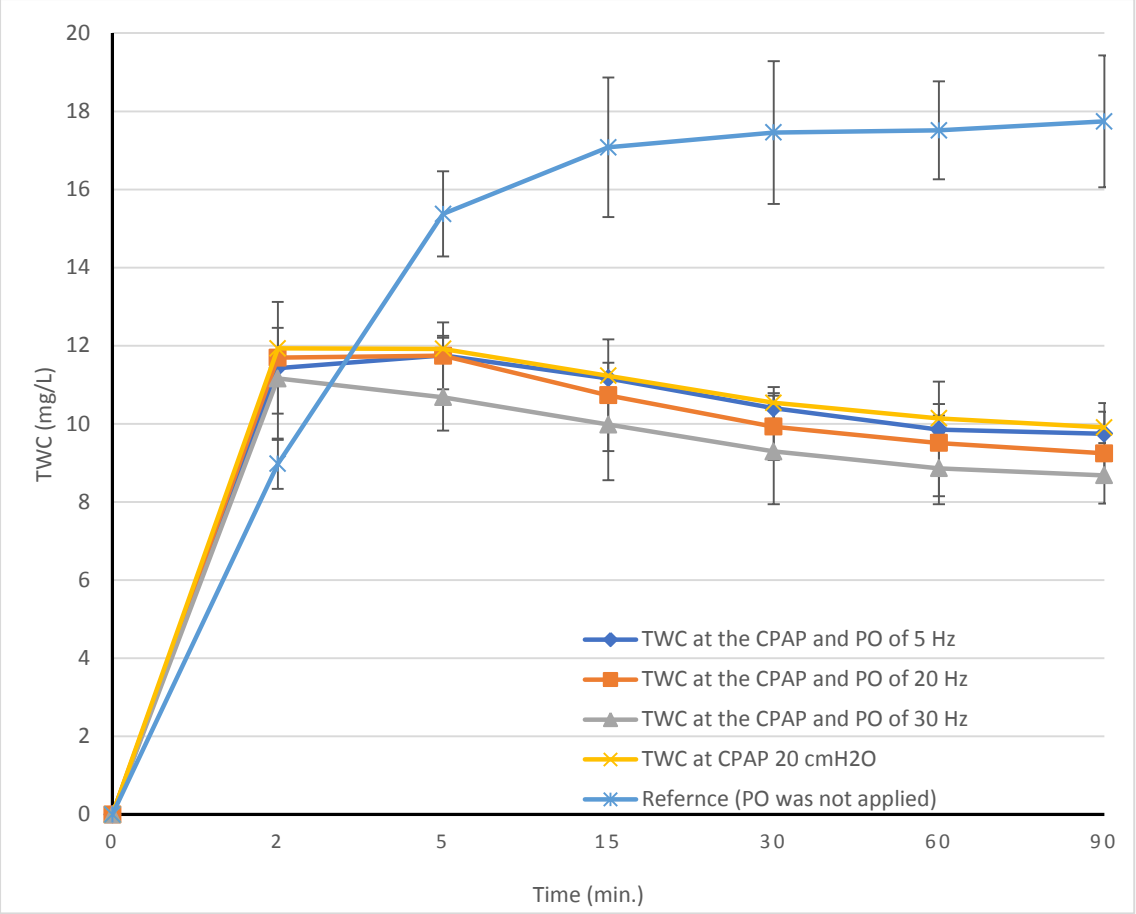


Figure 3.12. Total Water Content (TWC) of the air passing through the trachea, with values presented as mean ± SD for n = 7 samples.

Results of tracheal WF have shown an identical pattern to those data obtained when CPAP of 30 cmH_2O was applied (Figure 3.13). Data suggest that applying the PO at any frequency has no major improvement on the accumulative tracheal WF.

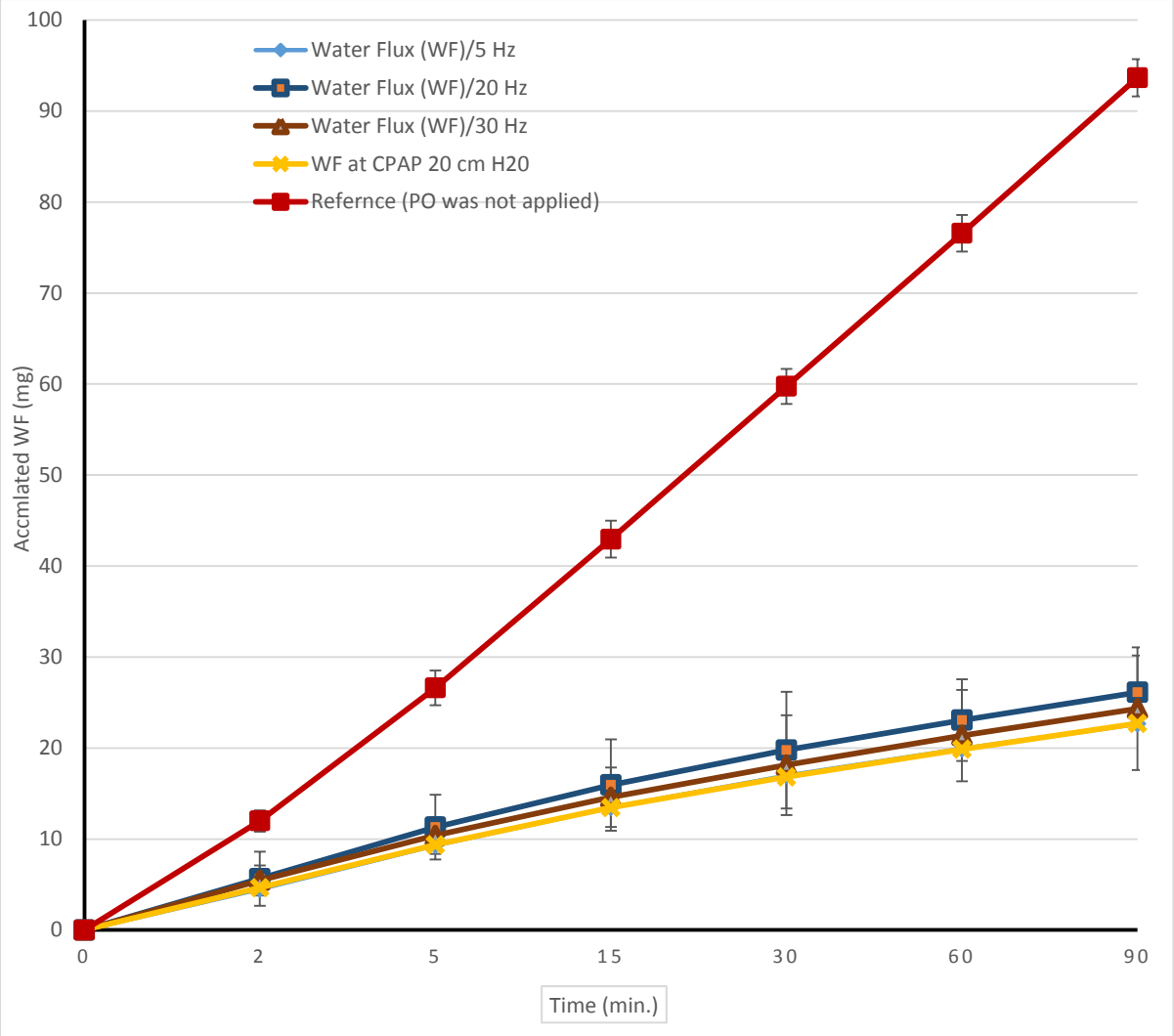


Figure 3.13. Accumulated tracheal Fluid depletion (WF), with values presented as mean ± SD for n = 7 samples.

3.8 Closure

This chapter has covered the description of equipment used in the study. Devices and the *ex vivo* experimental setup that facilitated study of the humidification parameters in bovine trachea were presented. Results obtained from tests of this study were also summarised and presented with brief expansions, however, all results are discussed in detail in the Chapter 4.

Chapter 4
Analysis of *Ex Vivo* Results

4.1 Introduction

Results of the *ex vivo* experimental setup were obtained from investigating various breathing conditions as presented in Chapter 3. In this part of this study, collected TWC values of the processed air and the amount of tracheal WF are furthered processed and presented in tables. A comparison between the outcomes of each experiment is established to determine the effect of applying various breathing modules on upper airway humidification. Furthermore, tracheal responses towards these investigated conditions are presented through measuring Transepithelial Potential Difference (TEPD). All experimental data are statistically analysed to confirm the significance of the data, and points are discussed comprehensively.

4.2 Analysis of the results

Quantification of humidity parameters of the processed air for normal breathing conditions, through applying various operating pressures of CPAP without and with PO waves were investigated. Conditions were in an *ex vivo* experimental setup. For normal breathing conditions, a lung simulator was facilitated, programmed and connected to the setup to simulate normal breathing conditions. Each investigation was performed using fresh bovine trachea and fresh physiological solution, repeated at least seven times in order to present statistically-powered results. Data is presented as mean ± SD.

4.2.1 Effect of the PO frequencies on the CPAP at 5 cmH_2O

The effect of applying variable conditions on the tracheal thermal capacity was investigated. For this purpose, three different PO frequencies of (5, 20 and 30 Hz) were selected. Data were then further processed to calculate the TWC and tracheal WF following the Equations 1 and 2. Results were then analysed, and standard deviation values were excluded to calculate the increase or decrease percentages compared with the results obtained at normal breathing conditions (without CPAP or PO). The following Equation was used to calculate changes in percentages:

$$\text{Change Percentage} = 100 \times \left[\frac{TWC\text{normal Breathing condition} - TWC\ Cpap/PO}{TWC\ normal\ Breathing\ condition} \right] \quad \text{(Equation. 3)}$$

Where:

Change percentage =refers to the difference within TWC of the processed air occurred due to the application of the PO at various frequencies, by comparing to TWC at normal breathing condition.

Table 4.1 shows a summary of the increased percentages in TWC of the processed air at investigated frequencies of applied PO. Major observation can be highlighted from the results. Data may confirm that applying CPAP at 5 cmH_2O has altered the humidification quality of the processed air compared to those obtained at the normal breathing conditions. Decreased percentages started at 2.2 % and continued to reach a drastic reduction after 90 min. (-18.6%). However, applying PO at any of the selected frequencies improved the humidification quality through promoting TWC of the processed air. A range of improvement percentages varied from 10.6 % up to a remarkable 68 %. The highest recorded improvement was observed when applying the high frequency value of 30 Hz. The lowest was observed at applying 5 Hz frequency. Applying the PO has maintained this improvement over the time of the experiment and promoted TWC values of the processed air. These observations may indicate the direct linear relationship between frequency values and TWC of processed air. Statistical analysis was performed using one-way ANOVA and results were significant ($p < 0.05$).

Table 4.1 Change percentages in TWC of the supplied air after CPAP at 5 cmH_2O with different breathing modules (n = 7).

Time (min.)	**% Change in TWC (mg/L) at CPAP of 5 cmH_2O**	**% Change in TWC (mg/L) at PO with amplitude of 1 V and frequency of**		
		5 Hz	**20 Hz**	**30 Hz**
5	3.9	10.6	14.1	23.1
15	- 2.2	19.2	28.1	39.5
30	- 8	25.2	38.5	50.3
60	- 13.1	35.5	48.0	59.2
90	- 18.6	42.7	53.7	68.0

Results for the amount of tracheal WF from ASL-facilitated passing air are depicted in Table 4.2 and clearly indicate two major observations. First, applying PO at the investigated frequencies altered tracheal capacity severely. A reduction in percentages is in the range of 38.4 to 48.2 % compared with tracheal WF results obtained at normal breathing conditions. A major reduction in tracheal capacity is observed with the applied PO at 5, 20 and 30 Hz and demonstrated a different pattern by improving altered tracheal capacity. The highest improvement of 78.85 % is recorded when applying a high frequency of 30 Hz. The lower improvement of 7.6% was recorded at applying 5 Hz frequency. Similarly, data suggest that there is a linear relationship between the magnitude of the frequency applied to the *ex vivo* setup and the accumulated tracheal WF. Statistical analysis of the results using one-way ANOVA confirmed the significance of these results with a $p < 0.5$.

Table 4.2 Change percentages within tracheal WF, as a result of CPAP at 5 cmH2O with different breathing modules (n = 7).

Time (min.)	**%Change in WF (mL) at CPAP of 5 cmH_2O**	**%Change in WF (mL) at a PO of 1 V and frequency of**		
		5 Hz	**20 Hz**	**30 Hz**
5	-38.4	7. 6	15.7	33.4
15	- 40.5	12.0	25.2	45.9
30	- 43.1	35.3	47.2	58.1
60	- 45.5	45.4	66.2	68.6
90	- 48.2	54.9	72.1	78.8

4.2.2 Effect of PO frequencies on the CPAP at 10 cmH_2O

At a higher operating pressure with CPAP of 10 cmH_2O, the results show a different pattern to earlier observations. As per Table 4.3, applying such conditions by CPAP has deteriorated the TWC of the processed air by reducing its value by 20.3% after 5 min. The TWC of the air deteriorated even further from 17.7 ± 0.4 mg/L to 10.3 ± 0.4 mg/L, representing a 41.9 % reduction of its original value at normal breathing conditions. Data shows an improvement within the humidification quality of the processed air attributed to the applying PO at investigated frequencies. Unlike previous experiments, improvement percentages were not as remarkable as those obtained at a lower CPAP pressure, and vary from 1.1 % to 20.5 %. The highest percentage was found associated with applying a higher frequency of 30 Hz, as it showed improved humidification quality of the processed air consistently. Surprisingly, medium and low frequencies showed fluctuations in these improvement percentages. Result may suggest the effectiveness of applying high frequency of the PO in improving humidification quality of the process air. Analysing obtained results has demonstrated the significance of these results with a $p < 0.05$.

Table 4.3 Change percentages in TWC of processed air at CPAP of 10 cmH2O and PO waves of various frequencies (n = 7).

Time (min.)	**%Change in TWC (mg/L) at CPAP of 10 cmH_2O**	**% Change in TWC (mg/L) at PO of 1 V and frequencies of**		
		5 Hz	**20 Hz**	**30 Hz**
5	-20.3	6.4	15.9	9.9
15	-28.9	3.6	7.3	8.6
30	-36.6	5.3	12.5	16.5
60	-40.1	1.1	10.0	20.9
90	-41.9	1.5	9.8	20.5

By comparing the result of applying PO and those obtained at applying CPAP alone, results show that tracheal response differed between the two conditions. Result in Table 4.4 reveals a drastic reduction within tracheal WF associated with applying CPAP at 10 cmH_2O. CPAP immediately altered tracheal WF by 58.9 % in comparison to those recorded at normal breathing conditions, and it deteriorated over the experimental time to 70.7 %. However, applying the PO at different frequencies mitigated the situation by inducing an improvement within the tracheal WF. Percentages of such improvement are remarkable and varied from 25.8 % to 51.5 %. The highest improvement was found to be associated with applying high frequency, while the lower is attributed to applying PO at 5 Hz frequency. Although improvement is observed during applying low frequency of 5 Hz during the experiment, both 20 and 30 Hz frequencies demonstrated a consistent improvement in tracheal WF.

Table.4.4. Change percentages within tracheal WF of the trachea, as a result of CPAP at 10 cmH_2O with different breathing modules (n = 7).

Time (min.)	% Change in WF (mL) at CPAP of 10 cmH_2O	% Change in WF (mL) at PO of 1 V and Frequency of		
		5 Hz	20 Hz	30 Hz
5	-58.9	26.5	36.2	41.3
15	-62.3	28.3	37.5	46.2
30	-65.7	27.9	45.8	50.5
60	-68.6	27.2	47.5	53.2
90	-70.7	25.8	44.8	51.5

4.2.3 Effect of PO frequencies on CPAP at 20 cmH_2O

Application of CPAP at 20 cmH_2O on humidification quality of the processed air was investigated. Table 4.5 summarises the results of TWC of the air at different conditions, and the increasing percentages were calculated by comparing results with those obtained at normal breathing conditions.

Table 4.5. Change percentages of CPAP at 20 cmH_2O and PO waves on TWC of processed air (n = 7).

Time (min.)	% Change in TWC (mg/L) at CPAP of 20 cmH_2O	% Change in TWC (mg/L) at PO of 1 V and frequency of		
		5 Hz	20 Hz	30 Hz
5	-22.8	-1.3	-1.4	-10.3
15	-34.3	-0.6	-4. 5	-11.1
30	-39.8	-1.3	-5.7	-11.8
60	-42.1	-2.9	-6.2	-12.6
90	-44.0	-1.7	-6.9	-12.4

Two major observations can be derived from the data. Firstly, applying CPAP at 20 cmH_2O reduced the TWC of the processed air by 22.8 % after 5 min., and the value is exacerbated to 42.1 % at the end of the experiment. This result suggests that the supplied air flow rate is well above tracheal thermal capacity. Another observation regarding the effect of applying PO waves confirmed that such waves at this pressure have altered air quality parameters of the processed air even more, and the proposed positive role of PO was no longer observed. Further reduction percentages in TWC of the processed air associated with applying PO frequencies varied from 1.3 % to 12.6 %, and the drastic reduction was attributed to applying higher frequency PO, while the lower reduction is associated with low frequency PO. Results obtained in this investigation were significant with a $p < 0.05$.

Table 4.6.Change percentages within tracheal WF as a result of CPAP at 20 cmH_2O with different breathing modules (n=7).

Time (min.)	**% Change in WF (mL) at CPAP of 20 cmH_2O**	**% Change of WF (mL) at PO of 1 V and frequency of**		
		5 Hz	**20 Hz**	**30 Hz**
5	-64.9	-0.4	0.9	1.4
15	-68.7	0.1	1.01	1.5
30	-71.8	0.9	1.5	1.9
60	-74.1	0.3	1.8	2.7
90	-75.8	-0.2	2.1	3.2

The amount of tracheal WF is indicated in Table 4.6 and shows that no major improvement was achieved while applying PO waves at the various investigated frequencies. Observations from investigating the effect of the PO when using CPAP at 20 cmH_2O pressure values indicates poor efficacy in comparison with the major improvements achieved at low pressure. This is likely due to the influence of the higher pressure applied by CPAP that overwhelmed the effect of the PO, and hindered the tissue relaxant effect induced by PO. Results were significant with a $p < 0.05$.

Results were processed further to determine the impact of applying each frequency of PO on humidification quality of the processed air and on tracheal WF. The first set of analyses was conducted on PO at a frequency of 5 Hz. Table 4.7 shows an improvement within the quality of air TWC achieved from applying 5 Hz PO. The range of improvement varied from 1.1 % to 42.7 % and was observed only at CPAP of low and medium operating pressures. Unlike previous observations, applying the CPAP at 20 cmH_2O diminished the positive impact, and altered TWC of the air slightly. Similarly, results supporting the claim of applying PO at 5 Hz may promote the tracheal WF when low and medium pressure of CPAP is applied. A range of improvement between 7.6 % to 54% was noted. The highest improvement was recorded when applying CPAP at 5 cmH_2O. However, the lowest was obtained at applying higher operating pressures by CPAP. Results may suggest a backward relationship between the TWC of air and tracheal WF on the one hand, and pressure applied by CPAP from other hand. PO at 5 Hz can only be beneficial to those parameters when applying low CPAP pressure.

Table 4.7. Change percentage in both TWC of the air and amount of Tracheal WF associated with applying PO at frequecny of 5 Hz (n=7).

At applying PO at an amplitude of 1 V and Frequency of 5 Hz						
Time (min.)	**Change Percentages in TWC (mg/L)**			**Change Percentages WF (mg)**		
	CPAP at 5 cmH_2O	**CPAP at 10 cmH2O**	**CPAP at 20 cmH2O**	**CPAP at 5 cmH_2O**	**CPAP at 10 cmH2O**	**CPAP at 20 cmH2O**
5	10.6	6.4	-1.3	7. 6	26.5	-0.4
15	19.2	3.6	-0.6	12.0	28.3	0.1
30	25.2	5.3	-1.33	35.3	27.9	0.9
60	35.5	1.1	-2.9	45.4	27.2	0.3
90	42.7	1.5	-1.7	54.9	25.9	-0.2

Processes were performed for applying PO at 20 Hz and are presented below. Table 4.8 shows an improvement within the quality of processed air. This improvement is thought to be attributed to superimposing PO at a frequency of 10 Hz. A range of improvement varied from 9.8 % to 53.7%, and was only observed at low and medium operating pressures of CPAP. No improvement is recorded at 20 cmH_2O pressure. Regarding the tracheal WF, results have confirmed the positive contribution of PO at this frequency in promoting the tracheal WF. Remarkably, the range of improvement was higher than lower frequency results, and was between 15.1 % to 72.1 %. Most importantly, applying this value of frequency was found to be beneficial even at CPAP of 20 cmH_2O. The highest improvement percentages are found at applying low pressure of CPAP, while the lowest percentages are associated with high pressure of CPAP. In general, data suggest the negative relationship between the applied pressure by CPAP and the scale of improvement within the TWC of air and tracheal WF that are attributed to applying PO at 20 Hz and 1 cmH_2O amplitude.

Table 4.8.Change percentage in both TWC of the air and amount of tracheal WF associated with applying PO at frequency of 20 Hz (n=7).

At applying PO waves at an amplitude of 1 V and Frequency of 20 Hz						
Time (min.)	**Change Percentages in TWC (mg/L)**			**Change Percentages WF (mg)**		
	CPAP at 5 cmH_2O	**CPAP at 10 cmH2O**	**CPAP at 20 cmH2O**	**CPAP at 5 cmH_2O**	**CPAP at 10 cmH2O**	**CPAP at 20 cmH2O**
5	14.1	15.9	-1.4	15.7	36.2	20.9
15	28.1	7.3	-4. 5	25.2	37.5	18.5
30	38.5	12.5	-5.7	47.2	45.8	17.5
60	48.0	10.0	-6.2	66.2	47.5	16.4
90	53.7	9.8	-6.9	72.1	44.8	15.1

Data are processed and presented in Table 4.9 for assessment of applying 30 Hz of PO. As per the results depicted in the table, the data shows a major improvement in quality of

processed air associated with applying the PO at a higher frequency. Improvement within the TWC of air is only observed at applying low and medium operating pressures of CPAP. The highest range of improvement percentages are between 23.1 % to 68.0 % and are found at applying low pressure of CPAP. Applying high pressure at 20 cmH_2O altered the humidification quality of the processed air by further reduction percentages between 10.3 % to 12.4 %. With no major deviation of previous observations, values of tracheal WF improved over all investigated pressure values of CPAP. The highest improvement percentages were between 33.4 % to 78.9 %, and were found at low CPAP pressure. The lowest improvement percentages were 11% to 7.3 %, and were associated with high CPAP pressure.

Table 4.9.Change percentage in both TWC of the air and amount of Tracheal WF associated with applying PO at frequecny of 30 Hz (n=7)

At applying PO waves at an amplitude of 1 V and Frequency of 30 Hz						
Time (min.)	**Change Percentages in TWC (mg/L)**			**Change Percentages WF (mg)**		
	CPAP at 5 cmH_2O	**CPAP at 10 cmH2O**	**CPAP at 20 cmH2O**	**CPAP at 5 cmH_2O**	**CPAP at 10 cmH2O**	**CPAP at 20 cmH2O**
5	23.1	9.9	-10.3	33.4	41.3	11.0
15	39.5	8.6	-11.1	45.9	46.2	8.5
30	50.3	16.5	-11.8	58.1	50.5	7.7
60	59.2	20.9	-12.6	68.6	53.2	7.7
90	68.0	20.5	-12.4	78.8	51.5	7.2

Overall results of Tables-1 to 9, reveal several major observations. First, applying CPAP at any operating pressure affected the humification quality of the processed air drastically. Reduction percentages within the TWC of the air that was associated with applying CPAP varied from 18.6 % to 44 %. The highest reduction percentages are found to be associated with applying the higher pressure of the CPAP. Results have shown that alterations associated with applying CPAP at any operating pressure may also extended to affecting the tracheal WF. It is reported that reduction percentages within the WF of trachea can be 38.4 %

up to 75.8 %.[144] The highest reduction percentages were associated with applying CPAP at 20 cmH_2O, and the lowest effect was associated with applying CPAP at 5 cmH_2O. Data suggest a backward relationship between CPAP and TWC values of the processed air and the Tracheal WF.

This can be explained by noting that applying CPAP at these operating pressures can increase the supplied airflow rate within the tracheal setup. Such conditions may develop within the tracheal lumen to accommodate air flux, and hence lead to the highest degree of muscle contraction by tracheal smooth muscles. This contraction in tracheal muscles (via cross-bridging) may develop an overlapping the epithelial cell within lining internal tracheal surface. Consequently, this overlapping can affect the epithelial cells role in maintaining the volume of the ASL and compensating for fluid depletion affected by ASL. Eventual restriction of tracheal thermal capacity by reducing tracheal WF values results. The highest pressure applied by CPAP leads to the severe overlaying that diminishes tracheal WF significantly, and vice versa.

Transepithelial Potential difference (TEPD) studies were performed to investigate tracheal response towards applying CPAP without and with PO. As, shown in Figure 4.1, results have confirmed fluid-depleted trachea are associated with applying CPAP. Results indicate that greater fluid depletion occurred when high pressure of CPAP was applied, leading to the fast response by tracheal tissue to compensate ASL depth. Consequently, tracheal response produced the highest potential difference of 37 ± 6.7 mV after 2 min. and it kept increasing to reach 67.8 ± 15.3 mV after 15 min.

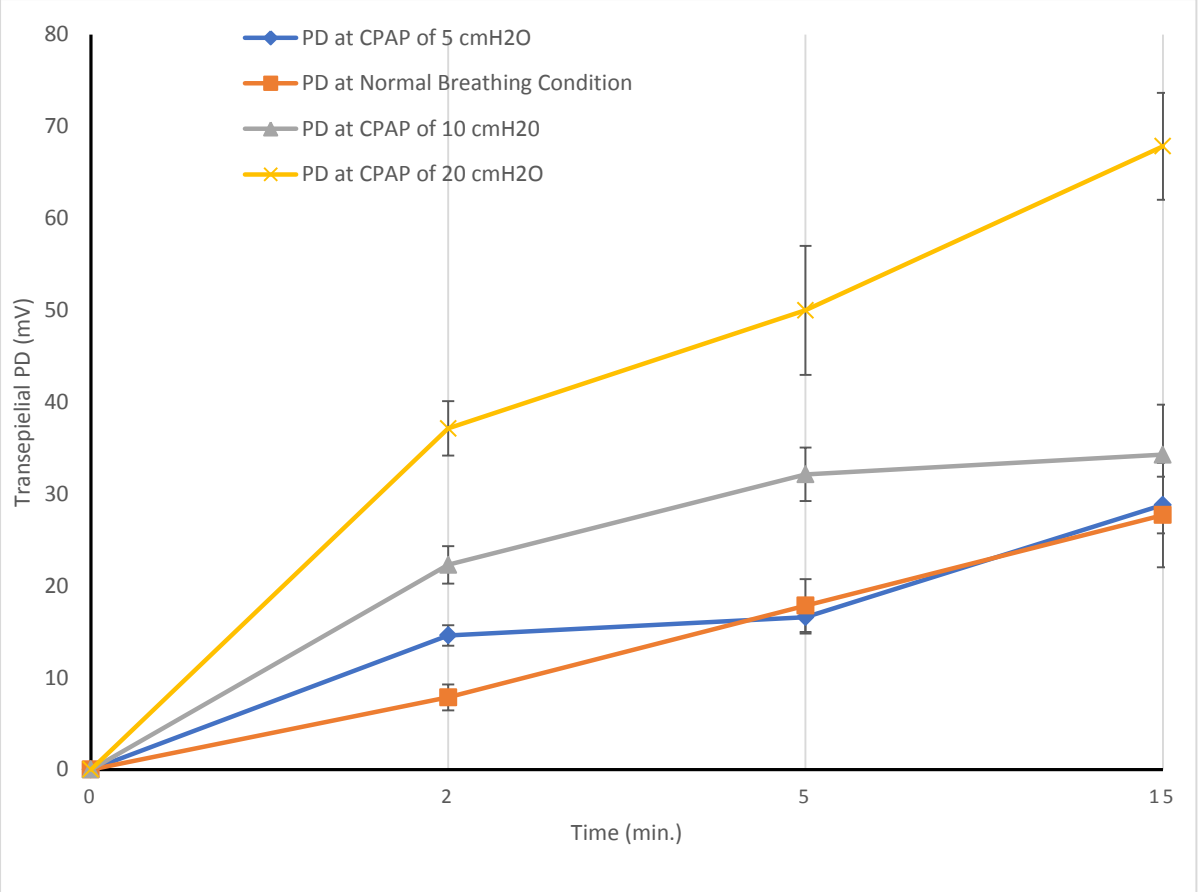


Figure 4.1. Transepithelial Potential Differences for Trachea at CPAP of 5 cmH_2O across different experimental conditions. Data presented as mean ± SD, n = 7.

Restoring the depth of the ASL of trachea is thought to occur through the well-known Active Ion-transport phenomena by which the leaky and water-ion permeable epithelia absorb Na^+ and secrete Cl^- ions simultaneously (Figure 4.2). During this process, the requirement for maintaining ASL isotonicity with surrounding plasma is considered as the main factor that governs the volume of ASL [209].

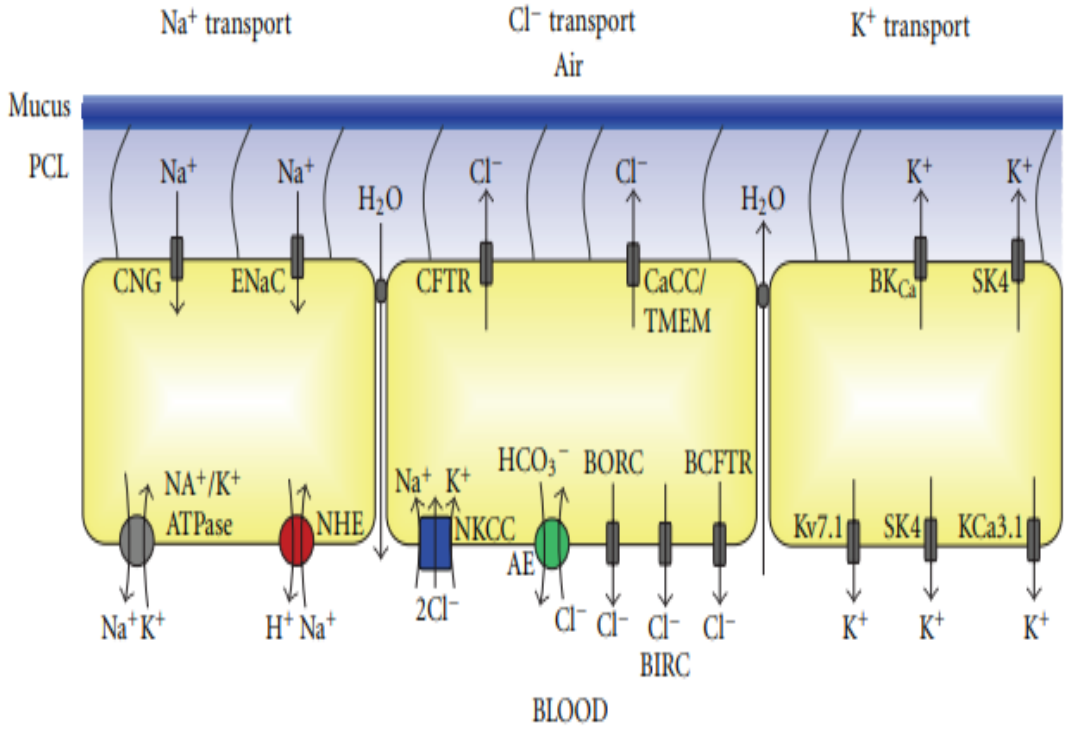


Figure 4.2. Mechanism by which the Active Ion-Transport phenomena across the trachea's tissue layers is carried out to compensate tracheal fluid depletion [209] .

Fluid depletion affects the ASL due to elevated air flow rates provided by CPAP. This current study has confirmed this observation, and therefore leads to disruption of the isotonicity status of the ASL. Trachea will respond with secretion of NaCl into the ASL via the epithelium. Such a movement will create an osmotic gradient, leading water to follow passively. Water flow will continue to restore the isotonicity of ASL. Consequently, an increase in the volume and depth of ASL [210] results, and it will promote the TEPD value as has also been confirmed within this study.

Another major observation is that applying PO at any of the investigated frequencies increased the TWC of the passing air significantly. These improvements were observed at CPAP of low and medium pressure values (5 and 10 cmH_2O). The highest increasing percentage of 68 % is found to be associated with applying PO at the higher frequency of 30 Hz. Results also reveal that optimal improvement caused by PO within TWC of air occurs at the tested low operating pressure of CPAP, while the lowest improvement is always attributed to applying high pressure. Accordingly, data may confirm that optimal benefits of

applying PO on improving the humidification quality of processed air can be achieved at 30 Hz frequency and at CPAP of 5 cmH_2O.

Results confirm that applying PO at any of the tested frequencies has improved the WF of trachea at any pressure value of CPAP. However, the highest improvement percentage of 78.8 % was achieved at applying PO at 30 Hz, in conjunction with the CPAP at 5 cmH_2O. Therefore, data suggest that in order to obtain the optimal improvement by applying PO, it has to be applied at high frequency with CPAP at a low operating pressure.

This remarkable improvement within the TWC and then within amounts of tracheal WF can be explained due to the impact of applying the selected PO waves to the ASL of the trachea. By applying PO superimposed on CPAP, generated waves may disrupt the dynamics of cross-bridge phenomena within tracheal muscle are disturbed. Therefore, PO applications mitigate the effect of shear forces created by CPAP on the ASL. Thus, the application helps the epithelial cells lining the trachea to perform their role of sensing and modulating the volume of the ASL effectively [211]. As a result, the volume of ASL can be maintained and compensation processes via active ion-transport can be performed adequately. Additionally, applying an intermittent type of force such as that provided by PO improved the TWC of the passing air, while applying a sustained flow rate pressure has deteriorated the TWC and caused fluid-depletion to the tracheal ASL.

Recording the TEPD at the conditions described in this study clearly indicated that applying high frequencies of PO (30 Hz) in combination with CPAP at 5 cmH_2O reduced the values of TEPD drastically. The results also show that applied PO waves at frequencies of 30 Hz improved corresponding TEPD values by 44.7%. Applying the lower frequency of PO has shown a subtle impact on the TEPD values, suggesting that complete muscle relaxation can only be achieved by applying higher frequencies at lower CPAP pressure.

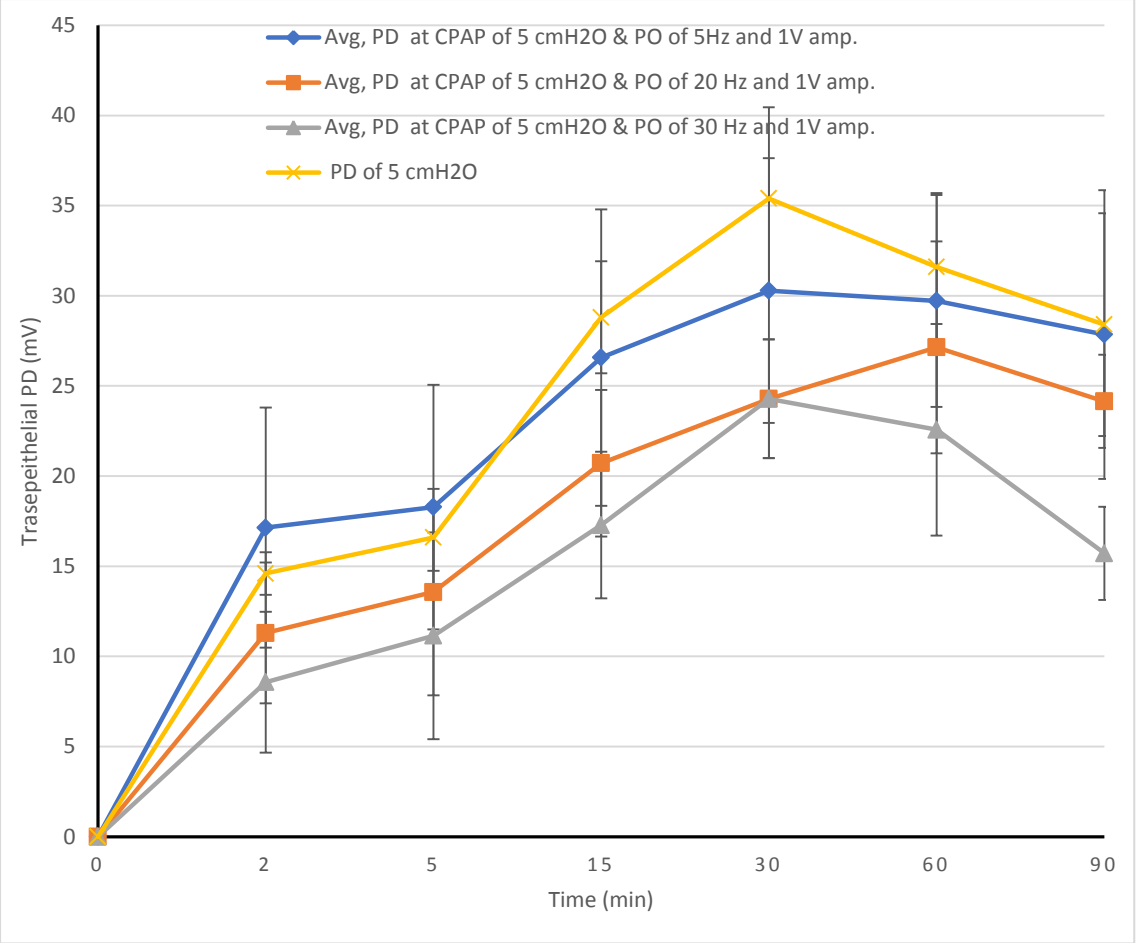


Figure 4.3. Transepithelial Potential Differences for tracheas at CPAP of 5 cmH_2O and different experimental conditions. Data presented as mean ± SD, n = 7.

Results were in the range of 38 - 14.8 mV, and statistical analysis for tracheal responses confirmed the significance among results with $p < 0.5$. Also, data from this study aligns with the value reported by others [202, 212]. Results also confirm the capability of the trachea to manage an increase of airflow rate supplied by CPAP at a lower operating pressure of 5 cmH_2O. Findings may also suggest the proper concept of experimental setup and its reliability.

The degree of muscle relaxation and hence restoration of ASL depth is a "CPAP pressure-dependent" parameter. Both the optimal muscle relaxation induced by the PO frequency and the ASL depth restoration can be governed by the value of frequency, and most importantly by the pressure applied by CPAP. The lowest pressure applied by CPAP will produce less muscle contraction and therefore, higher frequency is expected to induce an efficient muscle

relaxation, thereby contributing to optimal restoration of the ASL, and subsequent improvement both the TWC of processed air and the amount of tracheal WF.

4.3 Closure

This chapter presented the results obtained from assessment of humidification in the upper airway based on different applied breathing models. TWC values of processed air were supplied by CPAP at various investigated pressures and calculations indicated a negative relationship between the pressure applied by CPAP and the humidification quality of the processed air. Results also reveal that the tracheal WF is affected by applying elevated CPAP pressures. Facilitating the superimposed PO at various frequencies has improved humidification quality (TWC) of the processed air significantly. Such improvement is a "frequency-dependent increase". This improvement is also noticed in the amount of tracheal WF, suggesting that tracheal response towards different breathing models is variable, favouring PO at high frequency.

Chapter 5
Quantify Dryness associated with OSA following Clinical Trials

5.1 Introduction

Ex vivo results support the fact that applying CPAP at any operating pressure will drastically alter the tracheal performance of re-conditioned air. This part of the study is designed to investigate and quantify the dryness associated with the frequent use of CPAP. Quantifying the dryness is thought to be an important aspect since it may be facilitated to either improve current CPAP usage or it may be used to develop an alternative to CPAP. For example, by determining the dryness, quantitively, it can be used to redesign the conventional CPAP to deliver an exact amount of air-humidification necessary to eliminate the airway dryness experienced by OSA patients. Thus, it can reduce the size of the CPAP device and promoting patients' compliance. Also, it may be used to develop alternatives where water lost in air conditioning can be compensated for internally via stimulation of specific cells within the human body. The pressure oscillation technique represents a reliable alternative in which a positive impact has been confirmed by *ex vivo* studies in Chapter 4.

A clinical trial is thought to be beneficial to cover differences in the anatomical and tissue structures between the trachea alone (as in this study) and the oral cavity. To the best of our knowledge, no research has investigated the effect of applying CPAP and/or PO waves on the dryness of exposed tissues, nor on salivary flow rate.

Therefore, the main objectives of this part of the study are to investigate the effect of using CPAP without and with PO waves on dryness within the oral cavity. The clinical trials were conducted following approved ethical application. Evaluation of the dryness in relation to these investigated conditions was performed following spitting techniques. Samples of saliva were collected from participants exposed to a full session of CPAP without and with PO. The methodology of these trials is explained, results are further analysed to calculate the saliva flow rate, presented along with their statistical analysis, and followed by an explanation of the overall findings.

5.2 Clinical Study

5.2.1 Patient Recruiting Phase

Investigation of the effect of CPAP on the upper airways following *in vivo* techniques constitutes the second part of this study. All study procedures were first approved by the Health and Disability Ethics Committee, Ministry of Health. To perform this investigation, patients with OSA and having a long historical record of using CPAP are recruited by Fisher and Paykel Healthcare. Patients were selected from the company's records. Prior to performing the clinical trial, a Patient Information Sheet (PIS) and consent forms were prepared and sent to OSA patients within the company database Patients who expressed their interest to participate in this clinical trial were evaluated to exclude those who did not meet the study criteria. Eventually, selected patients were notified, and the clinical trial was scheduled at the sleep laboratory facility of Fisher and Paykel Healthcare. Quantification of the dryness associated with using CPAP on patients was performed following the spitting technique. To maintain consistency of performing the spitting technique and collection of saliva, protocols were developed based on previous research [213, 214].

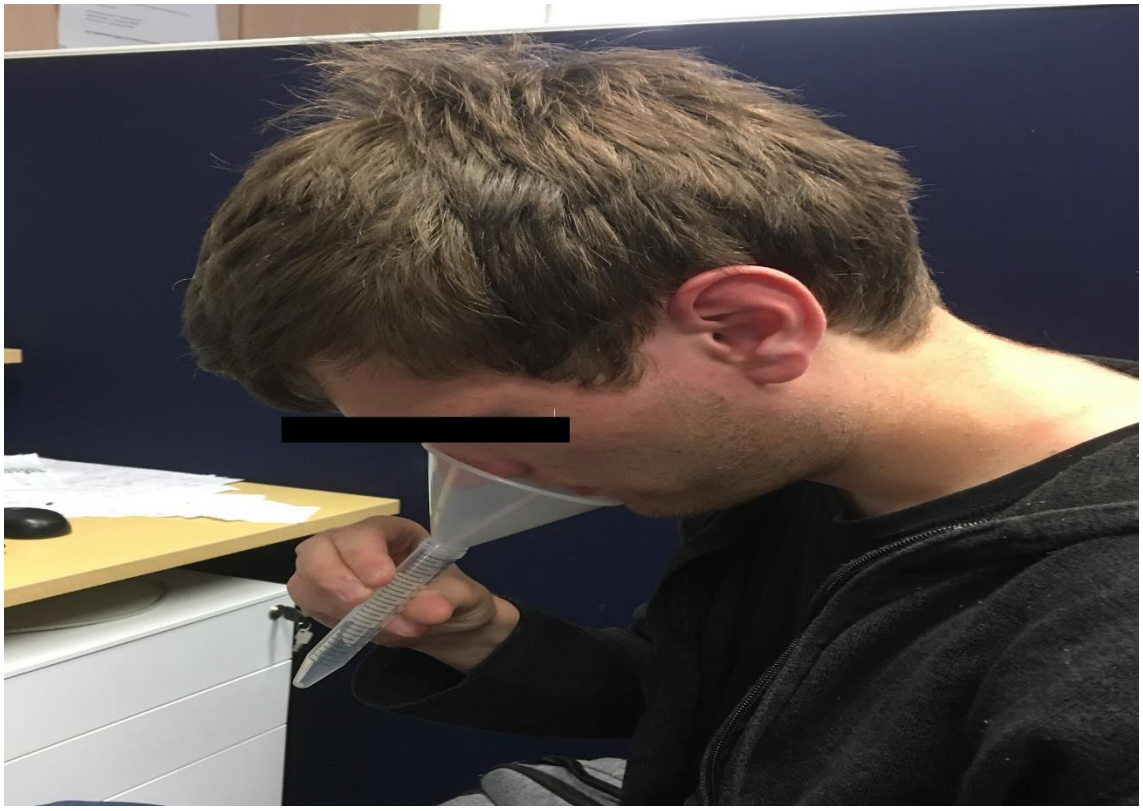


Figure 5.1. Sialometry used to collect saliva following spitting technique.

5.2.2 Anthropometrics

Results obtained from patients who participated in the clinical trial are presented against Body Mass index (BMI). Patient's BMI value is derived from measuring the patient's weight using an electronic scale, whereas the patient's height is recorded using a stadiometer. Equipment for measurements are located in the sleep lab of Fisher and Paykel Healthcare. Most importantly, each participant was assigned a code to identify them rather than personal details, thereby protecting their right to privacy, as shown in Table 5.1.

Table 5.1 Demographic details for clinical trial participants

Patient's ID	Age (Year)	Gender	Weight (kg)	Height (cm)	BMI
#1	62	M	106.8	175.5	34.7
#2	75	M	110	175	35.9
#3	79	M	91.6	172.8	30.7
#4	65	F	74	157.3	29.9
#5	67	M	112.8	182	34.1
#6	61	M	85.6	169.5	30
#7	70	M	99	175	32.3
#8	83	M	89	177.3	28.3
#9	61	M	89.9	173.5	28.3
#10	71	M	96.8	178.5	30.4
#11	76	M	155.6	178	49.1
#12	55	M	122	181	37.2
#13	71	M	83.1	173.5	27.6
#14	72	M	127.7	183	38.1
#16	84	M	176.5	99.1	31.8
#17	76	M	108.9	176	35.2
#18	46	F	150	171	51.3
#19	52	M	174	184	51.4

Calculation of patients' BMI is conducted via the website offers this service [215]. Due to the age-related restriction in the BMI calculation, patients above 70 years old are considered as 70 years old. In order to present high accurate, representative, and reliable results, patients with BMI $\geq$ 50 kg/m^2 are excluded. Also, due to the limited number of female patients , all females are excluded as well so study can produce results of high-risk patients.

5.3 Methodology

5.3.1 Quantification of Salivary flow rate protocols

Each participant in the clinical study was asked to follow the instructions as described below. Collection of saliva was conducted using sialometery, and each sample was coded to distinguish the collection of stimulated and non-stimulated saliva (Figure 5.1). Saliva collection was planned to occur before the CPAP session. Upon completion of the CPAP session, participants are asked to repeat the whole instruction set to collect saliva as per the protocol.

5.3.1.1 Before starting

1. Participants were advised to refrain from food, smoking, chewing gum, coffee, tea and beverages one hour before the test. Drinking water was allowed for participants.
2. Participants were seated and advised to rinse their mouth three times with deionised water and then relax for five min.
3. During the collection of saliva, participants were advised to minimise movements, not to swallow, and to keep their eyes open.
4. Participants were asked to swallow their saliva to void their mouth cavity of saliva.
5. During these processes, Sailometers (measured test tubes) were coded and pre-weighed for each participant.

5.3.1.2 Collection of unstimulated saliva

Participants were asked to follow the listed instructions:

1. Do not swallow during this trial, keep calm and eyes remain open

2. After one min., advise participant to collect the remaining saliva and spit it out into a plastic cup.
3. The first collection was discarded
4. Ask participants to collect saliva for five min. within their mouth cavity, without swallowing.
5. Upon the end of the five min. session, the participant was advised to lean their head forward and open mouth slightly and drain the saliva into a coded Sailometer, as shown in Figure 5.1.
6. Samples were then subjected to further processing.

5.3.1.3 Collection of stimulated saliva

1. Instruct participants to sit motionless.
2. Instruct participants to swallow to void their mouth of saliva.
3. A piece of neutral chewing gum was given to each participant and they were instructed to chewing at a pace of a metronome (approximately 70 strokes per min.).
4. Participants are advised to maintain the chewing pace without swallowing saliva, for one min.
5. At the end of one min., participants were asked to spit the collected saliva into a plastic cup.
6. The processes (above) were repeated for another min., and participants were asked to spit saliva out into the same plastic cup and discard it.
7. Participants were advised to repeat the procedure for three min. following the same instructions of no movement, no swallowing, and keeping eyes open.
8. At the end of the three-min. chewing session, participants were asked to spit the collected saliva into a coded Sailometer, without the gum.
9. Gum was discarded into a plastic cup
10. Samples were then processed further.

5.3.2 Application of the CPAP without and with PO

Protocols in sections 5.3.3.1, 5.3.3.2, and 5.3.3.2 were followed to collect saliva for each participant before and after applying CPAP to investigate the effect of CPAP on salivary flow rate (Figure 5.2).

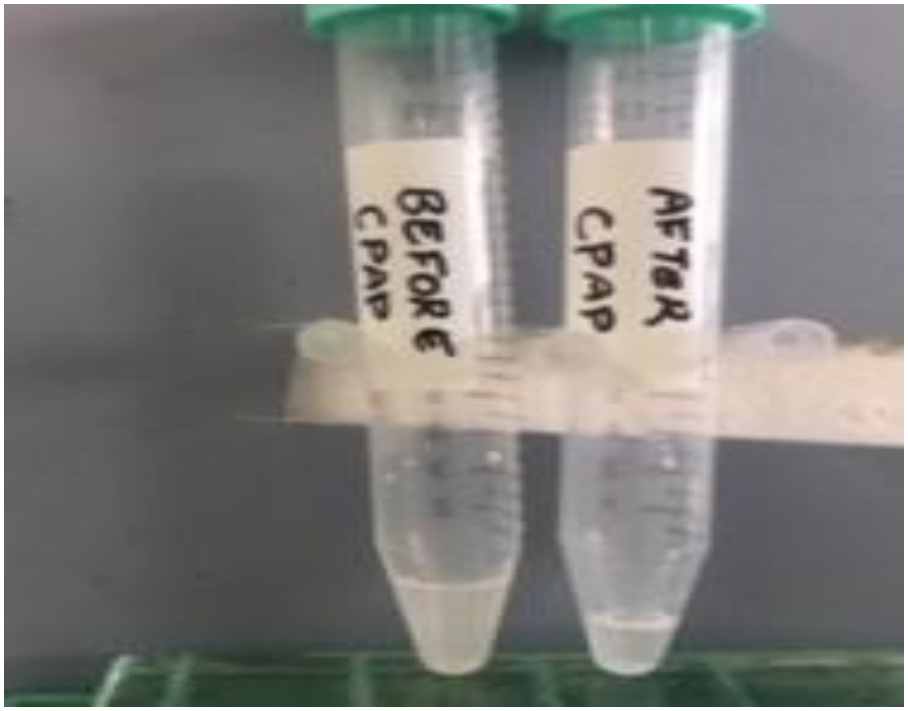


Figure 5.2. Samples of collected saliva for stimulated and non-stimulated saliva.

These protocols were followed by the same participants to assess salivary flow rate when CPAP with PO was applied for a full treatment session as shown in Figure 5.3

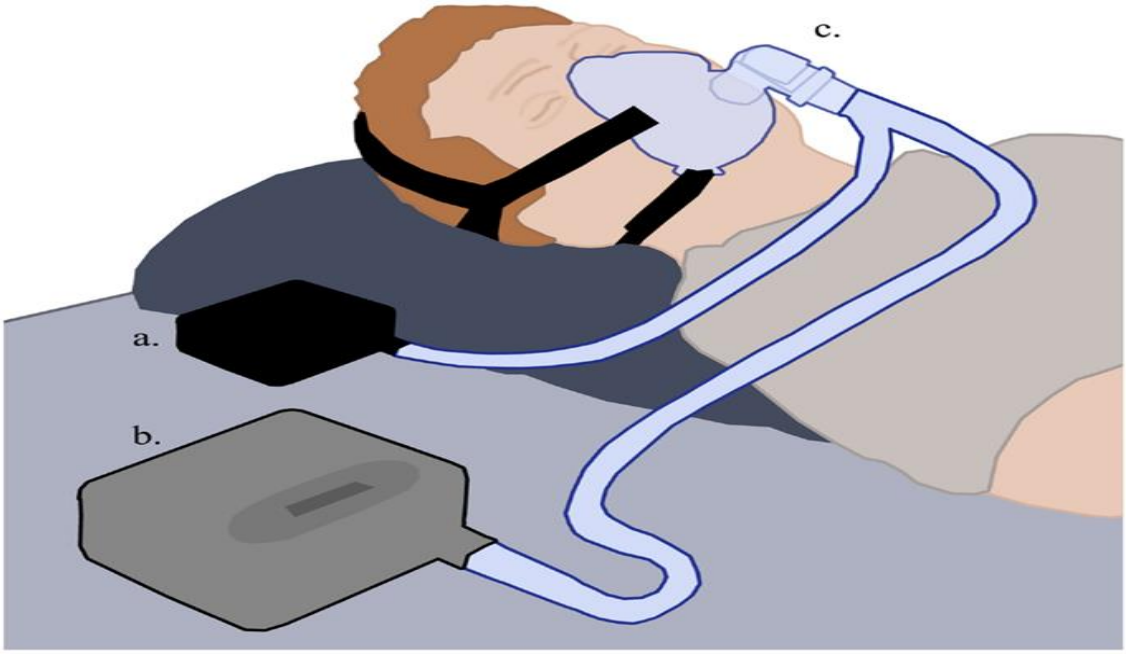


Figure 5.3. The positioning of the PO waves generator for waves(a) that are superimposed on the CPAP (b), and then delivered to patients with OSA through a mouth mask (c) [158].

5.3.3 Processing of saliva samples after collection

Samples of collected saliva from participants following the aforementioned producers are then processed following in below developed procedures based on reported research [216]

1. Each Sailometer was weighed individually and coded.
2. Net weight of saliva was calculated by subtracting post weight from the pre-weight.
3. Samples were then analysed to separate the solid and liquid content of saliva using a centrifuge (Hitachi, model 05 pr-22) set at 4000 rpm for 20 min.
4. Water content for each sample was pipetted out for weighing
5. Remaining solid matter weight was known (= $W_{total} - W_{\text{water content}}$)
6. To calculate the saliva flow rate (mL/min) (= $\frac{W\ stimulated}{3} + \frac{W\ unstimulated}{5}$)
7. Fill the records and write down all details as shown in Figure 5.4

Patient Code:			Date of trial :		
Collection Period	Method	Sailometer No.	Empty Tube (g)	Sample weight (g)	Salivary Flow rate ml/min
5	Non-stim. before the CPAP	1			
3	stim. before the CPAP	2			
5	Non-stim.after the CPAP	3			
3	Stim. after the CPAP	4			
5	Non-stim. before the PO	5			
3	Stim. before the PO	6			
5	Non-stim. after the PO	7			
3	Stim. after the PO	8			

Figure 5.4 Form of salivary flow rate calculation sheet for each participant

5.4 Results

Samples of collected saliva from various phases of this clinical trial were furthered analysed to measure the salivary flow rate of glands within the oral cavity. The clinical trial was performed as outlined in the Ethics application. Dryness associated with using CPAP was quantified, following the spitting technique. Saliva collection from participants was performed following the detailed instructions. The trial was able to provide two sets of data, as listed below:

- Pre- CPAP results that include:
    - Saliva produced without stimulation (at rest)
    - Saliva produced due to gum-based stimulation.
- Post-CPAP results that include:
    - Saliva produced without stimulation (at rest)
    - Saliva produced due to gum-based stimulation
- Post-application of CPAP with PO results that include:
    - Saliva produced without stimulation (at rest)
    - Saliva produced due to gum-based stimulation

5.4.1 Raw Data measurements

5.4.1.1 Saliva collection and analysing Pre- CPAP

Table 5.2. Salivary flow rate measurements and saliva composition for samples collected before CPAP.

Patient	Type of technique	Without the CPAP (Stimulated plus unstimulated saliva)			
		Solid Cont.(g)	Liquid Cont.(g)	Total(g)	Flow Rate (mL/min)
#1	**Non-stimulation***	0.02	0.205	0.225	0.045
	Stimulation**	0.495	0.861	1.356	0.452
#2	**Non-stimulation**	0.008	0.272	0.280	0.056
	Stimulation	0.172	3.146	3.318	1.106
#3	**Non-stimulation**	0.001	0.22	0.227	0.045
	Stimulation	0.059	1.251	1.311	0.437
#5	**Non-stimulation**	0.058	0.572	0.630	0.126
	Stimulation	0.195	1.899	2.094	0.698
#6	**Non-stimulation**	0.006	0.142	0.148	0.029
	Stimulation	0.004	1.180	1.184	0.395
#7	**Non-stimulation**	0.037	0.684	0.721	0.144
	Stimulation	0.04	5.947	5.987	0.662
#8	**Non-stimulation**	0.017	0.366	0.383	0.076
	Stimulation	0.054	1.401	1.455	0.485
#10	**Non-stimulation**	0.004	0.666	0.670	0.134
	Stimulation	0.005	2.116	2.121	0.707
#11	**Non-stimulation**	0.015	0.355	0.367	0.074
	Stimulation	0.080	2.500	2.580	0.860
#12	**Non-stimulation**	0.018	0.602	0.620	0.124
	Stimulation	0.020	2.251	2.271	0.757
#14	**Non-stimulation**	0.158	0.502	0.660	0.132
	Stimulation	0.228	3.069	3.297	1.099
#16	**Non-stimulation**	0.109	0.621	0.73	0.146
	Stimulation	0.393	1.026	1.419	0.473
#17	**Non-stimulation**	0.087	0.383	0.47	0.094
	Stimulation	0.853	2.558	3.411	1.137

*Referred to the quantity, composition, and saliva flow rate that collected from patients at resting condition
**Referred to the quantity, composition and saliva flow rate that collected from same patients using chewing gum following protocol outlined in Section 5.3.1.3

Table 5.2 shows a summary for saliva weight obtained from participants before exposure to an 8 hours session of CPAP treatment. Samples were collected at resting conditions and through the stimulation technique using a piece of neutral chewing gum. Due to the nature of these samples and trials, no standard deviation was applied. As shown in Table- 2, the saliva

flow rate values were ranging from 0.045 mL/min to 1.137 mL/min. Also, these flow rate values varied significantly. The highest values were obtained following stimulation techniques. The lowest was achieved at resting conditions. The compositions of the saliva samples were analysed following the protocol mentioned above in Section 5.3.3. Data revealed that the produced saliva was mostly liquid in nature.

5.4.1.2 Saliva collection and analysing after applying CPAP

Samples of saliva collected from participants after a full session of CPAP treatment were analysed to determine the contribution of saliva produced via un-stimulation and those from stimulation methods. Samples were also analysed to measure saliva flow rate. Table 5.3 shows a summary of data and it also confirms variation within saliva flow rate among participants. The lowest flow rate value was found to be around 0.043 mL/min, coming from saliva collected at resting conditions. The highest rate was 1.117 mL/min obtained following stimulation technique. In general, the saliva flow rate collected in this part of the study demonstrated a reduction within the produced saliva rates.

Table 5.3. Salivary flow rate measurement and saliva composition for samples collected after CPAP

Patient	Type of technique	With the CPAP (Stimulated plus unstimulated saliva)			
		Solid Cont.(g)	Liquid Cont.(g)	Total(g)	Flow Rate (mL/min)
#1	**Non-stim.***	0.015	0.2	0.215	0.043
	Stimulation**	0.15	0.851	1.001	0.334
#2	**Non-stim.**	0.058	0.177	0.235	0.047
	Stimulation	0.069	4.365	4.434	0.301
#3	**Non-stim.**	0.036	0.157	0.193	0.038
	Stimulation	0.065	0.994	1.059	0.353
#6	**Non-stim.**	0.025	0.109	0.134	0.027
	Stimulation	0.076	0.932	1.008	0.336
#7	**Non-stim.**	0.033	0.096	0.128	0.026
	Stimulation	0.048	1.986	2.034	0.678
#8	**Non-stim.**	0.114	0.281	0.395	0.079
	Stimulation	0.075	1.0117	1.092	0.364
#10	**Non-stim.**	0.055	0.618	0.0673	0.135
	Stimulation	0.097	0.525	0.622	0.207
#11	**Non-stim.**	0.076	0.620	0.696	0.139
	Stimulation	0.052	1.012	1.064	0.355
#12	**Non-stim.**	0.034	0.397	0.431	0.086
	Stimulation	0.029	1.579	1.608	0.536
#14	**Non-stim.**	0.121	0.284	0.405	0.081
	Stimulation	0.101	2.083	2.184	0.728
#16	**Non-stim.**	0.12	0.68	0.8	0.160
	Stimulation	0.416	02.355	2.77	0.923
#17	**Non-stim.**	0.048	0.211	0.26	0.052
	Stimulation	0.396	1.845	2.241	0.747

*Referred to the quantity, composition, and saliva flow rate that collected from patients at resting condition
**Referred to the quantity, composition and saliva flow rate that collected from same patients using chewing gum following protocol outlined in Section 5.3.1.3

5.4.1.3 Saliva collection and analysing after applying the CPAP with PO

Table 5.4. Salivary flow rate measurements and saliva composition for samples collected after CPAP with PO.

Patient	Type of technique	With the CPAP and PO (Stimulated plus unstimulated saliva)			
		Solid Cont.(g)	Liquid Cont.(g)	Total(g)	Flow Rate (mL/min)
#2	**Non-stim.**	0.054	0.180	0.222	0.047
	Stimulation	0.097	1.094	1.191	0.397
#3	**Non-stim.**	0.046	0.14	0.193	0.039
	Stimulation	0.030	1.338	1.368	0.456
#5	**Non-stim.**	0.045	0.474	0.519	0.104
	Stimulation	0.059	5.739	5.798	1.933
#7	**Non-stim.**	0.002	0.099	0.101	0.020
	Stimulation	0.055	1.424	1.479	0.493
#9	**Non-stim.**	0.015	0.142	0.157	0.031
	Stimulation	0.051	1.896	1.946	0.648
#11	**Non-stim.**	0.036	0.029	0.065	0.013
	Stimulation	0.159	1.431	1.59	0.53
#12	**Non-stim.**	0.110	0.50	0.61	0.122
	Stimulation	0.599	2.731	2.330	1.11
#13	**Non-stim.**	0.121	1.113	1.235	0.247
	Stimulation	0.158	1.136	1.293	0.431
#14	**Non-stim.**	0.106	0.454	0.56	0.112
	Stimulation	0.555	2.365	2.92	0.973
#16	**Non-stim.**	0.229	1.652	1.881	0.376
	Stimulation	0.079	2.287	2.367	0.789
#17	**Non-stim.**	0.087	0.383	0.470	0.094
	Stimulation	0.433	1.907	2.34	0.78

*Referred to the quantity, composition, and saliva flow rate that collected from patients at resting condition
**Referred to the quantity, composition and saliva flow rate that collected from same patients using chewing gum following protocol outlined in Section 5.3.1.3

The last part of this study was conducted after applying a treatment session of CPAP alongside PO. Summary data for the saliva flow rate collected from participants is presented in Table 5.4. Samples were analysed, and the composition of the saliva is included in the table. As per the results, the saliva flow rate varied similar to the same observations in the

previous sections. The highest value recorded in this trial was 1.933 mL/min, and it was well above other rates.

5.5 Analysis and Brief Discussion

Another important aim of this part of the research was to investigate the occurrence of dryness and quantifying it within OSA patients classified as regular users of CPAP. The effect of CPAP on the salivary flow rate was assessed following the *in vivo* technique, where a clinical trial was designed in order to collect saliva from participants. Various techniques of saliva collection have been reported and investigated comprehensively [213]. The draining method, suction method, swab method, and spitting technique are prime examples. The latter was selected due to the simplicity and it doesn't interfere with any stimulation to the salivary flow rate which may affect the accuracy of results. Another important aspect that the study took into consideration was that quantification of saliva flow rate was conducted on measuring the whole salivary flow rate rather than measuring the saliva flow rate from the three major salivary glands. The selection was made based on the fact that the entire salivary output is well represented by the oral cavity conditions. Basically, saliva is produced predominantly from three paired main glands (parotid, submandibular and sublingual). [217]. The mechanism by which these glands producing saliva it similar, however, some are producing saliva at resting conditions while others require a stimulation. In order to cover the two different types of saliva, a piece of chewing gum was selected to trigger this stimulation mechanically.

5.5.1 The long-term effect of applying CPAP

The effect of CPAP is assessed by comparing the study's results of participants before applying CPAP and normal salivary flow rate reported in previous research [218, 219] as it was outlined in the methodology and results sections.

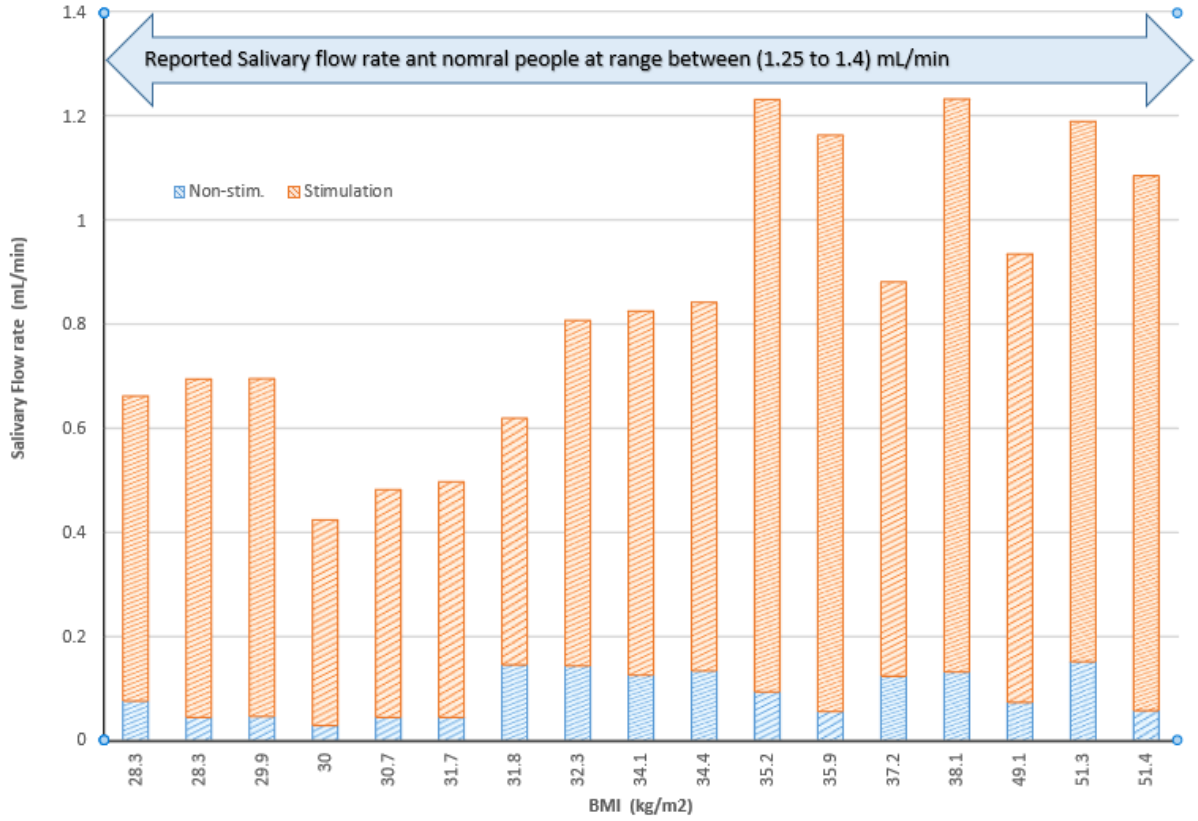


Figure 5.5.Total salivary flow rate and composition of saliva for participants collected before applying CPAP

Figure 5.5 shows the total salivary flow rate for participants before exposing them to any treatment. Flow rates ranged between 0.424 mL/min to 1.23 mL/min. By comparing these salivary flow rate values with those reported for normal people, results confirm that a major reduction has occurred with the salivary flow rate of participants. The percentage of reduction values were calculated by subtracting salivary flow rates values obtained from each participant before applying CPAP with the average values reported by others, following the given equation.

$$\text{Change Percentage} = 100 \text{ X} \left[\frac{Flow\ Rate\ (h) - FlowRate\ (c)}{Flow\ Rate\ (h)} \right] \qquad \text{(Equation. 4)}$$

Where:

Flow Rate(h) = Salivary flow rate of healthy people obtained from reported research

Flow Rate (c) = Total Salivary flow rate derived from clinical trial samples.

Table 5.5. Change percentages altered the salivary flow rate of participants before applying CPAP

Patients	BMI (kg/m^2)	% Change in salivary flow rate without the CPAP
8	28.3	51.9
6	30.0	69.1
3	30.7	64.9
1	31.7	63.8
16	31.8	54.9
7	32.3	41.3
10	34.4	38.8
17	35.2	10.5
2	35.9	15.4
12	37.2	35.9
14	38.1	10.4
11	49.1	32.1

Table 5.5 shows that the reduction percentage in total salivary flow rate of participants varied among participants and it ranged from 10.4 % to 69.2 %. The highest salivary flow rates were obtained from participants with high BMI of 35.2 kg/m^2 and above. The lowest salivary flow rates were collected from participants with BMI equal to or below 34.4 kg/m^2. Overall observations clearly indicate that all participants have a salivary flow rate below the normal values.

Analysing the contribution of both stimulation and non-stimulation salivary flow rates from the obtained total values was conducted. The contribution varied, and the mean of non-stimulated salivary flow rate was 0.09 ± 0.04 mL/min, which represented approximately 11.1 % of the total salivary flow rate. Results of the stimulation salivary flow rate showed it had a major contribution to the total salivary flow rate with 0.74 ± 0.25 mL/min and this value represented 88.9%.

5.5.2 Effect of applying the CPAP

Saliva samples were collected immediately after the end of an 8-hours treatment session of CPAP. Results were analysed and plotted against BMI (Table 5.6).

Table 5.6. Change percentages within salivary flow rate of participants after full session treatment using CPAP.

Patients	BMI (kg/m^2)	% Change in salivary flow rate with the CPAP		
		Non-stim. Salivary flow rate	Stim. Salivary flow rate	Total salivary Flow rate
8	28.3	-3.3	24.9	21.1
6	30	9.5	14.9	14.5
3	30.7	14.4	19.39	18.8
1	31.7	4.4	26.2	24.2
16	31.8	-9.6	10.6	5.8
7	32.3	82.2	-2.3	12.8
10	34.4	0	70.3	59.4
17	35.2	44.7	34.3	35.1
2	35.9	17.1	72.9	70.2
12	37.2	30.44	29.2	29.4
14	38.1	38.64	33.8	34.4
11	49.1	-88.14	58.8	47.2

Results depicted in Table 5.6 shows that after applying full session treatment using CPAP, participants experienced a major deterioration in their salivary flow rate, which was already considered as low. Reduction percentage values were calculated following Equation-3, and reduction within the total salivary flow rate ranged from 5.81 % to 70.2 %. The highest decreasing percentages within the salivary flow rates were found with participants of BMI higher than 34.4 kg/m^2. The lowest percentages were found within participants with BMI of equal to or lower than 32.8 kg/m^2. Although results have shown a consistent reduction mainly associated with stimulation-based saliva, results reveal non-consistency over the salivary flow rate produced by the non-stimulation technique. One-way ANOVA analysis of the salivary flow rate collected before and after CPAP treatment shows a significant effect for the BMI of participants, with a $p < 0.5$.

The mechanism by which reduction within salivary flow rate has occurred is not certain and there is not enough research regarding this topic. However, we try to explain this phenomenon from the anatomical perspective. Salivary secretion is primarily produced by

three glands, the parotid, submandibular and sublingual glands. Altogether, they contribute 90 % of the fluid within the oral cavity [217].

Salivary fluid is first secreted by the acinar cells and emptied into ductal cells that are responsible to modify, regulate and convey it to the surface of mouth [220]. Basically, the mechanism of transportation by which acini fluid flows depends on vectorial transportation ion movement, based on the difference in isotonicity of the fluid, and salivary fluid will be hypotonic when it is delivered to the mouth [221]. Thus, applying elevated air pressure through using CPAP may evaporate most of the saliva, specifically the serous content, due to reconditioning the excessive air flow rate. Consuming the serous content of saliva on the surface of the mouth will disrupt the hypotonicity status of the saliva within the oral cavity, making it dry. Dehydration of 8 % of body water content can lead to a significant reduction in the salivary flow rate [213]. Consequently, applying CPAP may lead to significant local dryness within the upper airway, potentially causing a severe effect on the salivary flow rate, and thereby reducing it drastically, as results have confirmed.

5.5.3 Effect of applying the CPAP with PO waves

After the confirmation of dryness associated with using CPAP by OSA patients, the study proposed applying PO waves superimposed on CPAP as an alternative to overcome the dryness feeling. Assessment was performed following the spitting technique and the procedures were carried out as outlined in the methodology. Samples of saliva were collected from participants after exposure to a full session of 8 hours treatment with CPAP and PO. Results were compared with those obtained from applying only CPAP.

Results depicted in Table 5.7 show a major improvement in the salivary flow rate recorded from participants after CPAP and PO treatment sessions. Improvement percentages are in the range of 0.49 to 1.39 mL/min, representing an increasing percentage between 9.4 % up to 129 %. The highest improvement was recorded within the salivary flow rate of the participants with a BMI of 29.9 kg/m^2, whereas the lowest was obtained with participants of 49.1 kg/m^2 Statistical analysis for salivary rates after applying CPAP and after applying CPAP with PO found that this improvement within the salivary flow rate and BMI of participants is significant with $p < 0.5$.

Table 5.7. Change percentages within salivary flow rate attributed to applying full treatment session of CPAP with PO.

Patients	BMI (kg/m^2)	% Change in salivary flow rate with the CPAP and PO		
		Non-stim. Salivary flow rate	Stim. Salivary flow rate	Total salivary Flow rate
8	28.3	212	18.4	53.
6	30	44.3	35.7	36.4
16	31.8	-30	129.9	86.1
7	32.3	-21.3	27.3	27.1
17	35.2	80.8	4.5	9.4
2	35.9	0	32.2	27.9
14	38.1	38.2	33.8	34.2
11	49.1	-90.7	49.4	9.9

Saliva composition data were recorded and clearly indicated that the major contribution of 90.7% is collected following stimulated saliva technique. While the remaining saliva (9.3%) was contributed from the non-stimulated techniques, consistent with previously observed results.

The mechanism by which this remarkable improvement was achieved is not clear. Nevertheless, the study suggests that applying PO waves at high frequency and an amplitude above 1 cmH_2O can stimulate specific muscles within the UAW such as recruitment of the genioglossus muscle and the sternomastoid. The mechanism by which a muscle's recruitment occurred is thought to be through stimulation of the receptors at the UAW and thus trigger reflux activation of the genioglossal muscles. Muscle recruitment has been assessed by recording the EMG activity of these muscles [154]. Physiologically, the genioglossal muscle, as all other intrinsic and three other extrinsic muscles of the tongue, are innervated by the cranial nerve (XII) which is known as the hypoglossal nerve (Figure 5.10). Recruitment of these muscles will produce a tongue movement that may act as a mechanical stimulation to the salivary glands to provide an increase in salivary flow rate.

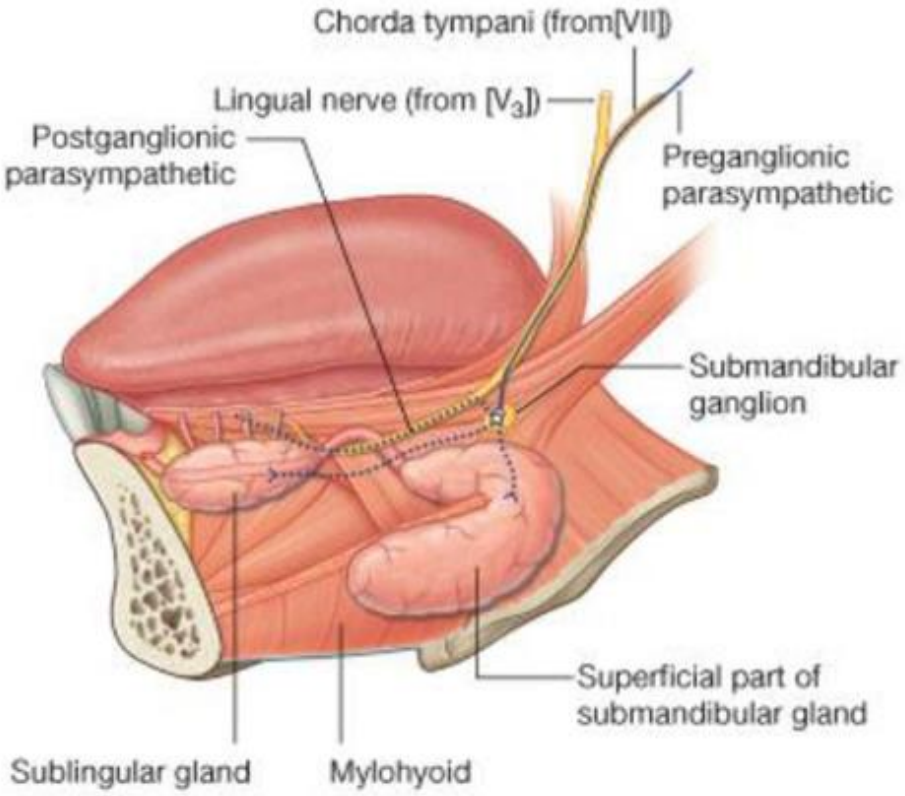


Figure 5.6. Hypoglossal nerve and salivary glands [222].

Results of participants show differences within their salivary flow rate, suggesting that participants respond to the superimposing PO differently. This variability indicates that other factors may contribute to this inconsistency. Factors like differences in patient's age and weight may have an important effect on the salivary flow rate. Furthermore, health conditions for participants that may require taking medications which cause dryness as a side effect, and hence leads to the poor salivary flow rate, may also play a role.

By analysing improvements recorded as a result of adding the PO waves to CPAP treatments, results have confirmed that major contributions of this improvement within the salivary flow rate are from the saliva that is produced via stimulation processes using neutral chewing gum. As per Table 5.4, results show around 90% of the saliva collected after applying the PO waves was stimulated-based saliva. This study's finding is in alignment with the speculation regarding the occurrence of reflexes triggering the hypoglossal nerve that innervates both intrinsic and some of the extrinsic muscles. Another major finding within this part of the study was about the composition of the collected saliva. Samples were further analysed

following the same protocol described in Section- 5.3.3, and results have confirmed that more than 93% of the saliva collected after applying the PO waves was serous-based saliva. These findings may suggest that a major contribution of saliva has been produced from the parotid pair of glands since their saliva was described as purely serous saliva.

5.6 Closure

As per the study objective, the results presented in this chapter were obtained from assessing and quantifying dryness associated with applying CPAP in OSA patients. Calculation of salivary flow rate of participants shows a severe dryness attributed to the frequent and regular use of the CPAP by patients with OSA. Facilitating the superimposed PO with CPAP as alternative OSA treatment has improved salivary flow rates in participants significantly. These observations are in alignment with *ex vivo* results obtained in Chapter 4 and emphasise the positive role of PO in OSA treatment. Further analysis with comprehensive discussion of these results is presented in the following chapter.

Chapter 6
Electrical Stimulation - Alternative Therapy to Treat Patients with OSA

6.1 Introduction

Another alternative that may be used to replace CPAP treatment is developing a bioimprinted polymeric implant. Such an implant can be inserted into the hypoglossal nerve that innervates the hypoglossal muscle which is responsible for tongue protrusion. By electrically stimulating the hypoglossal, the tongue will be protruded and repair the loose tone that caused narrowing within the UAW. In the last few years hypoglossal nerve stimulation techniques have been developed and investigated comprehensively [129, 161, 223-230]. However, insertion of a foreign object like the metallic electrode may trigger the body's immune system following the well-known Foreigner Body Response (FBR) phenomenon that develops from persistent inflammatory stimuli, eventually leading to deteriorating long-term efficacy [231-233]. This study hypothesises that developing a bioimprinted polymeric implant can address these issues adequately. Inserting such an implant having the same morphological features of the cells of surrounding tissues, is thought to has various advantageous characteristics. First, it will be recognised as a familiar object, not as a foreign body, and hence does not trigger the immune system. Also, due to its morphology it’s thought to improve bio-integration with surrounding tissues. Consequently, it may improve long-term performance without causing any health issues.

To build such an implant, various Polypyrrole (PPy) formulations will be investigated and optimised to select the high-performance formulae. This selected PPy nanocomposite will be utilised to build a polymeric scaffold. For this purpose, three different polymeric materials will be investigated and characterised following different techniques to select a suitable candidate. Eventually, a bio-compatibility and electrical stimulation characterisation will be performed to confirm their tolerance and determine the suitable potential to deliver electrical stimulation.

6.2 Methodology

This methodology section is divided into three main sections: the first presents the synthesis of Polypyrrole (PPy), chemicals utilised, and characterisations. The second part explains building bioimprinted implants, including chemical information, characterisation, and most importantly testing the bio-compatibility. The third part investigates the electrical stimulation impact on cellular proliferation.

6.2.1 Polymer fabrication

6.2.1.1 Chemicals and instruments

Pyrrole was purchased from Merck-Aldrich (Germany, 99% pure) and was purified by distillation under reduced pressure at room temperature using a lab distillation setup. Purification of Py was conducted in a dark room as it is a product known to be sensitive to both air and light. The distilled pyrrole was protected through a nitrogen blanket and stored in an amber glass container in the refrigerator at 4 °C until it was used in the polymerisation process. Ferric sulfate ($Fe_2(SO_4)_3$, M.wt. 399.88 g/mol), ferric chloride ($FeCl_3$; M.wt. 162.204 g/mol), and ammonium persulphate (APS)($(NH_4)_2S_2O_8$, M.wt. 228.18 g/mol) were used as oxidising agents and were of analytical grade. Surfactant (Kolliphor P188) was purchased from Sigma-Aldrich. All other reagents and chemicals were of analytical grade and were used as received. The quality of water used within all processes was distilled water produced by Millipore preparation (Milli-Q water, Millipore, USA).

6.2.1.2 Polypyrrole (PPy) synthesis

PPy was synthesised following a chemical polymerisation technique. Due to the simplicity of the method, ability to produce fine PPy powder, and affordability of equipment, the process was performed within our laboratory [234-236]. Briefly, the polymerisation process was performed using three oxidants of FeCl, $Fe_2(SO_4)_3$, and $(NH_4)_2S_2O_8$ with the following concentrations of 1M (16.2 g) of anhydrous $FeCl_3$ or 1 M (20 g) of $Fe_2(SO_4)_3$ or 1 M (452 g) of $(NH_4)_2S_2O_8$ initially prepared for the subsequent reactions. Oxidants were prepared at room temperature, and under constant agitation (600 RPM) using a magnetic mixer. The process was conducted until the oxidant was dissolved completely. An aqueous solution of pyrrole was then prepared by adding 1 M (3.45 g) of freshly distilled pyrrole into 50 mL of

distilled water. Dilution was conducted under a blanket of nitrogen. Mixing of both solutions took place following the ratio of 1:2.3 (monomer: oxidizing agent). The reaction is classified as exothermic, and so the addition of pyrrole solution into the oxidising agent solution was dropwise and under a constant stirring rate of 800 rpm till the amount of pyrrole solution was consumed. The polymerisation phase was carried out for four hours at a speed of 500 rpm, then the solution was kept unagitated under nitrogen and protected from light overnight. The precipitated black PPy powder was then filtered out using filter paper under vacuum. The precipitated, filtered powder was then washed with methanol and distilled water repeatedly to remove any impurities. The filtered and washed black powder of PPy was then dried for two days at room temperature.

6.2.1.3 Polymerisation of Polypyrrole with Surfactant

The previous version of PPy was furthered developed by using Kolliphor P188 (Pharmaceutical grade) as a dopant. This material was selected due to its versatile properties, as it is considered to be a non-ionic surfactant with comprehensive application in biomedical applications, in addition to its functional role of being a dopant in PPy synthesis [237]. Addition of Kolliphor P188 is thought to promote the bio-compatibility and improve the electrical conductivity of PPy. The polymerisation process was similar to the method previously discussed. However, the addition of a surfactant was conducted by mixing the pyrrole solution with surfactant at a percentage of 10% by weight. All other polymerisation methods were carried out following the same process as described above.

6.2.2 Developing Bioimprinted polymeric substrate

The synthesised PPy doped with surfactant nanocomposites were used to develop conductive bioimprinted polymeric substrates. The process was performed following a protocol based on previous research [238, 239].

The human epithelial Caco-2 cell line is selected to be used as a model for human epithelium in this study as it is been used widely by various researches, easily maintained and availability within out lab [240, 241]. Cells were purchased from ATCC® HTB-37, as frozen cells. Cells were then stored in liquid nitrogen until they were used. Cells were thawed in a water bath at 37 °C for 1 min. Cells were resuspended in pre-warmed Dulbecco's modified

eagle's medium (DMEM) with 1000 mg/L Glucose supplemented with 5% fetal bovine serum (FBS), and 1% Penicillin-streptomycin -Glutamine (PSG), and cultured in T-75 growth flasks. Cells were incubated in 5% CO_2 atmospheric conditions using a standard incubator (Heracell CO_2 incubator, Thermo Fischer). Cells were left for 9 days to grow; during which time the medium was discarded and replaced with prewarmed fresh medium every two days to replenish nutrients and maintain appropriate pH conditions for cell growth.

During this period cells were examined using microscopy periodically to confirm their viability and proper growth. When cell growth reached above 80% confluence, cells were then washed with PBS, thoroughly, trypsinized to lift the attached cells, and counted under microscopy (Carl Zeiss, Jena model Telaval). Cells were then seeded into 5 cm plastic petri-dishes at a density of 50,000 cells per cm^{-2} and incubated for 24 hours at 5% CO_2 atmospheric condition to allow cells to attach to the plate surface. After 24 hours, the cell media from flasks was aspirated and cells were washed with cold PBS solution, twice, followed by pouring 4% paraformaldehyde fixative solution (Thermo Fischer fixative solution 4% formaldehyde) over the cells for fixation. Flasks with fixation solution were left for 45 min. to allow the solution to fix cells effectively. Upon cell fixation, flasks were then rinsed with cold PBS, gently, followed with a final rinse using distilled water. Flasks were then dehydrated with ethanol solution to remove moisture and left at 4 °C overnight.

To prepare bioimprinted conductive polymeric substrates, three polymeric materials of polydimethylsiloxane PDMS (Sylgard, Dow Corning, USA), Chitosan (Low Molecular weight, Sigma Aldrich) and Gelatin (Difco, Granulated, DB, USA), were selected. Preparation of the PPy polymer scaffold was carried out depending on the type of polymer. For Chitosan: a 3% chitosan solution was made using 2.5 % acetic acid aqueous solution. The solution was used to suspended 500 mg of PPy, using an ultrasonicator probe (Vibracell, Sonic, and material, USA) for 10 min. at 40 % power. The resulting solution was poured onto the petri-dish where the cells were fixed. Amounts of 4-6 grams were poured to obtain a conductive polymeric membrane having a thickness of 2 mm. samples were then moved into the vacuum chamber to remove all air bubbles trapped within the polymeric substrate and to improve replication accuracy. Upon completion, samples were left overnight.

The preparation of Gelatin, as a solution of 15%, was first prepared following the abovementioned steps. Preparation of PDMS first used the synthesised PPy powder (500 mg), resuspended in a sufficient quantity of acetic acid, followed by mixing with 20 g of PDMS

using an ultrasonication probe for 1 hour. The resulting mixture was then cured with an agent at a ratio of 10:1 (v/v). The mixture was then poured gently onto a petri-dish flask, and then placed under a vacuum to remove air bubbles; hardening steps were performed at 60 °C for 3 hours using a vacuum oven.

6.2.3 Investigate the electrical stimulation on the cell proliferation

Cellular proliferation responses towards electrical stimulation was investigated using a customised conductive bioimprinted polymeric substrate inserted in a 5 cm plastic petri-dish developed for this purpose. Developing this petri-dish was performed following the same steps mentioned earlier. To conduct this investigation, a DC potential was delivered to cells attached on the polymeric substrate through the conductive bioimprinted polymeric substrate, using two built-in metallic electrodes. These two electrodes were made of copper and inserted on the edges of the petri-dish, and the polymeric solution was poured onto the samples. These electrodes were connected to insulated thin copper wires, then connected to a power generator, via alligator cables (Figure 6.1).

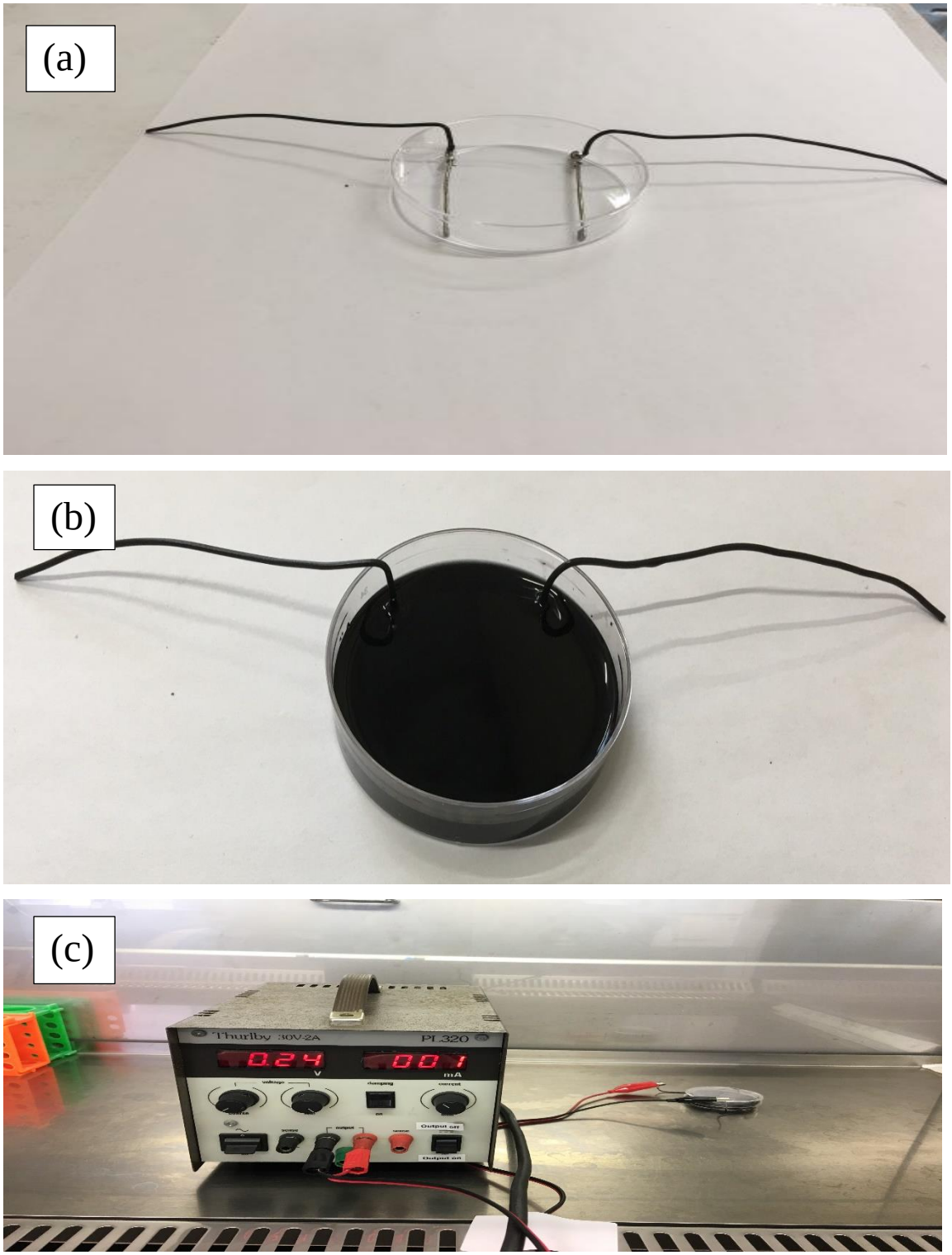


Figure 6.1 steps of developing Bioimprinted conductive polymeric substrate; (a) the layout of two electrodes sit inside a customised 5 cm petri dish; (B) pouring the polymeric material; and (c) connecting the conductive substrate to power signal generator

The resulting petri-dishes were then cleaned in a sonication bath with distilled water for 30 min. and left to dry at ambient temperature overnight. Prior to use for cell culture, samples

were UV-sterilised for 2 hours. Cells were then seeded at a density of 50,000 cells per cm^{-2} on the PPy/polymeric substrate and incubated for 24 h to allow adhesion to surface. Electrical stimulation of DC with values of 150, 600, and 1000 mV/mm was applied for 5 h to the cells through the conductive bioimprinted polymeric substrate. Upon completion of the electrical stimulation session, flasks were then incubated for an additional 24 h.

6.3 Statistical analysis

data are presented as mean ± SD of the three readings. The data were analysed using t-test and one-way ANOVA to measure the p-value. The p-value was considered significant if the p-value was < 0.05.

6.4 Characterisation

6.4.1 Characterisation of polymer

6.4.1.1 FTIR -Spectrometry

To confirm the formation of PPy, developed samples were tested for structural and optical characteristics using Fourier transformation infrared (FTIR) Nicolet iS 10 FT-IR spectrometers (Thermo Fischer Scientific). The aim of this test was to analyse the chemical bonding of PPy and to confirm the link of the surfactant to the PPy chain. Samples were tested directly without further preparation, and each sample was scanned for 64 scans using a spectral resolution of 16. All spectra within the range of 400 - 4000 cm^{-1} for prepared PPy nanocomposites were recorded, coded, and saved into a dedicated file on PC.

6.4.1.2 Morphological studies

Morphological features for both the developed formulations of PPy powder and the thick film were studied by Scanning Electron Microscope (SEM), using a Hitachi field emission scanning electron microscope (SU70 FE-SEM, Japan). Prior to being tested, PPy powder samples were first glued to the sample holder using double-face adhesive tape. To improve the conductivity of the sample and produce high-resolution images, samples were coated with an ultrathin layer of gold using Ion Sputter coater (MC1000, Hitachi Inc.). Images obtained from the test were then saved within dedicated files.

6.4.1.3 Electrical Conductivity studies

The electrical conductivity behavior as a function of temperature for the developed PPy powder formulated from various oxidising agents was conducted following the well-known four-point probe technique. Also, the electrical conductivity of the bioimprinted polymeric implant was evaluated following the same technique and at room temperature. Therefore, two different protocols were prepared with different procedures. For PPy powder, the nanocomposite of PPy powder was first compressed into circular-shape pellets with a diameter of 13 mm through applying a pressure of 12 MPa using a proper compression tool) (Figure 6.2(a)).

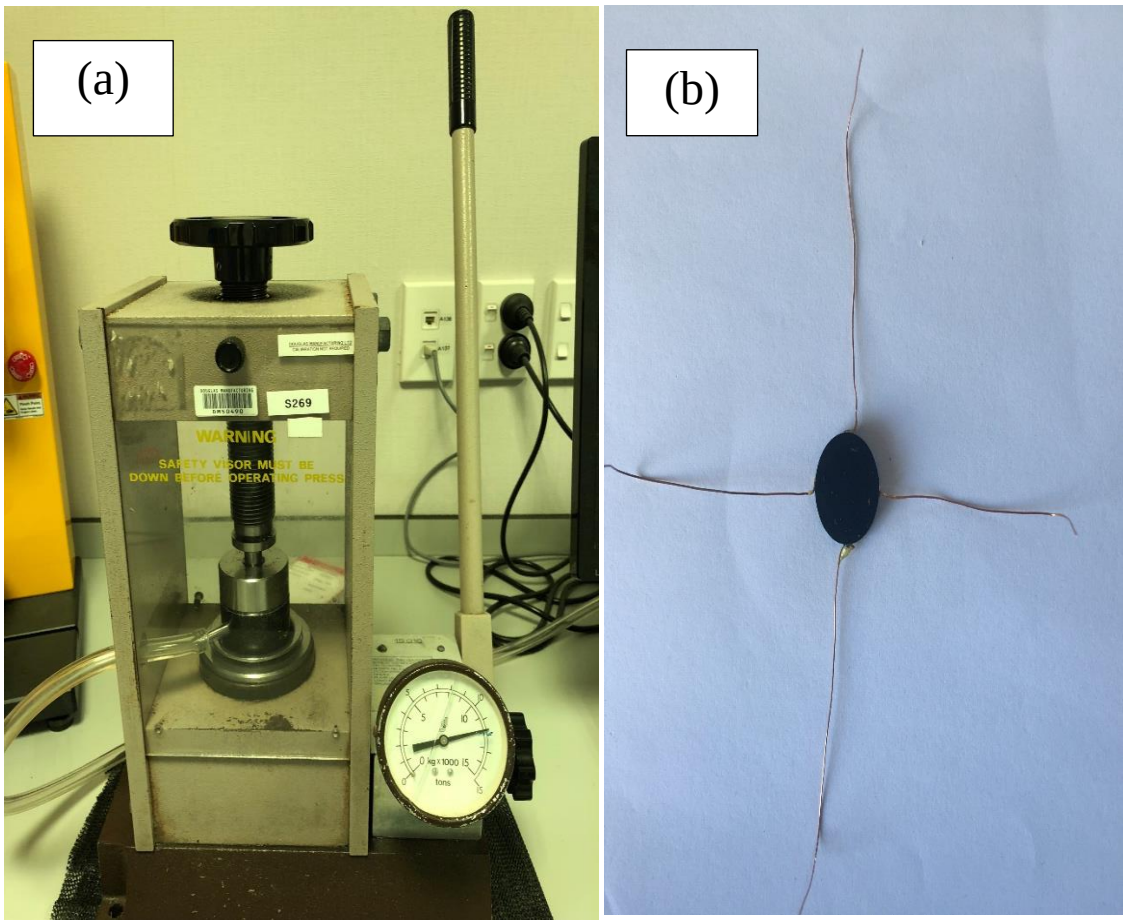


Figure 6.2 procedure to pressed PPy nanocomposite into disk-shape using a proper compression tool (a); the obtained disks were then connected with copper wire to measure conductivity(b)

Upon completion of compression, the thickness of PPy disks was measured and recorded. Four pieces of thin copper wire (0.05 mm) were then glued to the edges of the disks of PPy, as shown in Figure 6.2(b) using a silver conductive wire glue-paste adhesive to improve

electrical conductivity. Wires were placed in such a way to ensure equal distances between these wires. PPy disks were then placed within the thermo-controlled chamber and the terminals were connected to a digital precision multimeter (Tektronix, DMM 4040), placed outside as depicted in Figure 6.3. Resistivity as a function of applied temperature was recorded, and further processed to calculate the electrical conductivity [242, 243].

Figure 6.3. Experimental setup to measure the resistivity of PPy powder as a function of temperature

6.4.2 Characterisation of Bioimprinted implant

6.4.2.1 Morphological studies

Obtained implants that developed following bioimprinted procedure outlined in Section 6.3 were tested to confirm replication accuracy within implant' surface. Similarly, the preparation of samples is identical to what has been described in Section.

6.4.2.2 Electrical conductivity study

Following the same four-probe resistivity measurement technique, the electrical conductivity for the developed PPy bioimprinted implants was conducted at room temperature as shown in Figure 6.4. Values were then processed to calculate the conductivity of various polymeric substrates.

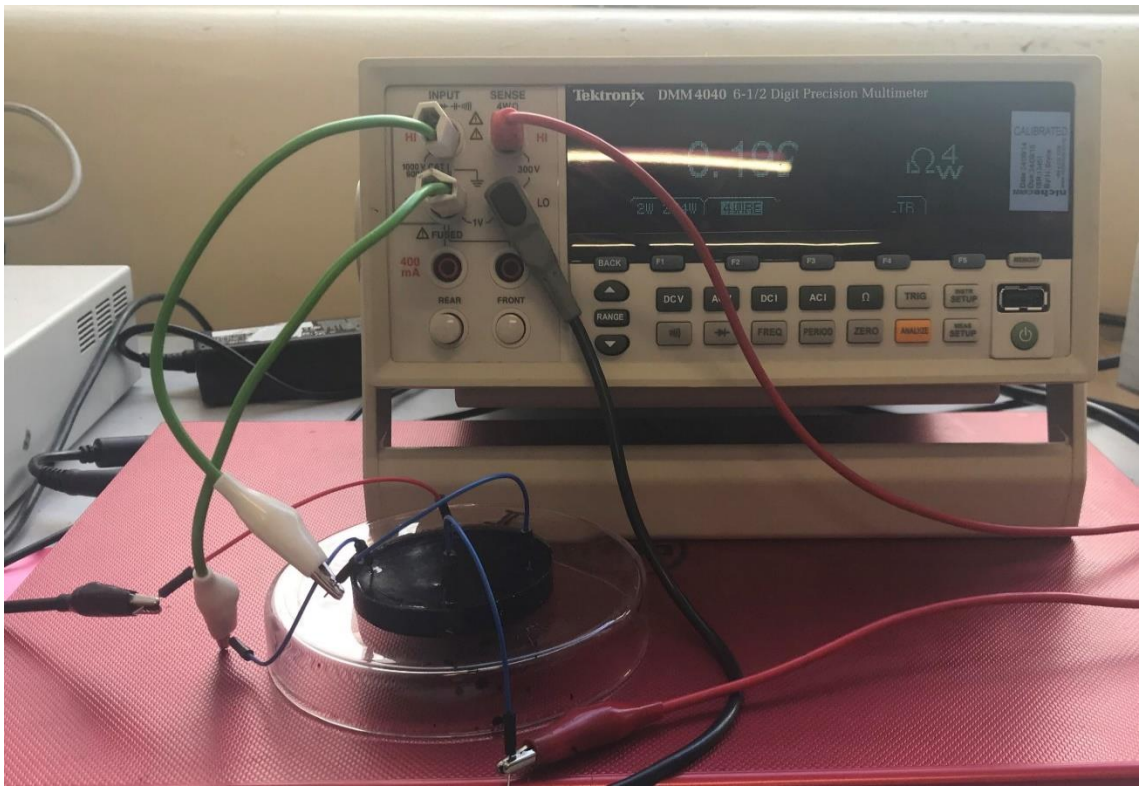


Figure 6.4 Resistivity measurement for Bioimprinted polymeric implant following the four-point probe technique

6.4.2.3 Biocompatibility test

After cells were exposed to electrical stimulation with various potential gradient values, cells were assayed using Flow Cytometry (Beckman Coulter, MoFlo XDP). In order to prepare

samples, the medium was first aspirated, and cells were then washed with PBS gently, treated with trypsin to lift the cells which were attached to the polymeric substrate. Cells were counted and resuspended in 200 µL binding buffer of the kit (Annexin V FITC and PI). Samples were incubated with 10 µL of Annexin and 5 µL of propidium iodide (PI). Mixing of these samples was performed to ensure homogeneity and improve binding of solutions to the cells. Dilution of samples was conducted with 500 µL of binding buffer, followed by mixing to improve homogeneity and binding. All these preparations were performed under sterile conditions and samples were kept in an ice bath to avoid oxidation. Samples were analysed immediately.

6.5 Results and Brief Discussion

6.5.1 Identification using FTIR

Various experimental parameters may have a direct impact on PPy quality. Studies have reported the effects of type of solvent and temperature during processing, as well as pH and types of oxidising agents used on PPy [244-247]. In this study, PPy was synthesised using three different oxidising agents: ferric III oxide, ferric sulphate, and ammonium persulphate. The resulting samples were characterised using FT-IR spectroscopy to confirm their identity. The FT-IR spectra of PPy powder within the wavelength region between 500 to 4000 cm^{-1} was presented in Figure 6.5. The recorded spectra show the characteristic band attributed to the C-C asymmetric stretching vibration at 1440 cm^{-1}, while the band at 1522 cm^{-1} is assigned to a ring-stretching mode of PPy. Both bands represent the fundamental vibration of the PPy ring and thus confirm the identity of PPy. The spectra may reveal that there was a shift in the bands within the developed formulations, this trivial shift may occur due to the degree of surfactant doping within the PPy preparations. This difference may lead to affect the delocalisation of π-electrons within the skeletal vibration of the PPy ring. Also, the strong bands at 1134 cm^{-1} may correspond to the doped PPy chain, suggesting that surfactant has been doped in the PPy chain efficiently. Other bands that appeared in the spectra at 1640 cm^{-1} may be assigned to the C=N bond, while the 1003 cm^{-1} reading may corresponding to the C-H and N-H deformation [248]. In addition, the observed band at 3411 cm^{-1} corresponds to the N-H bond [249].

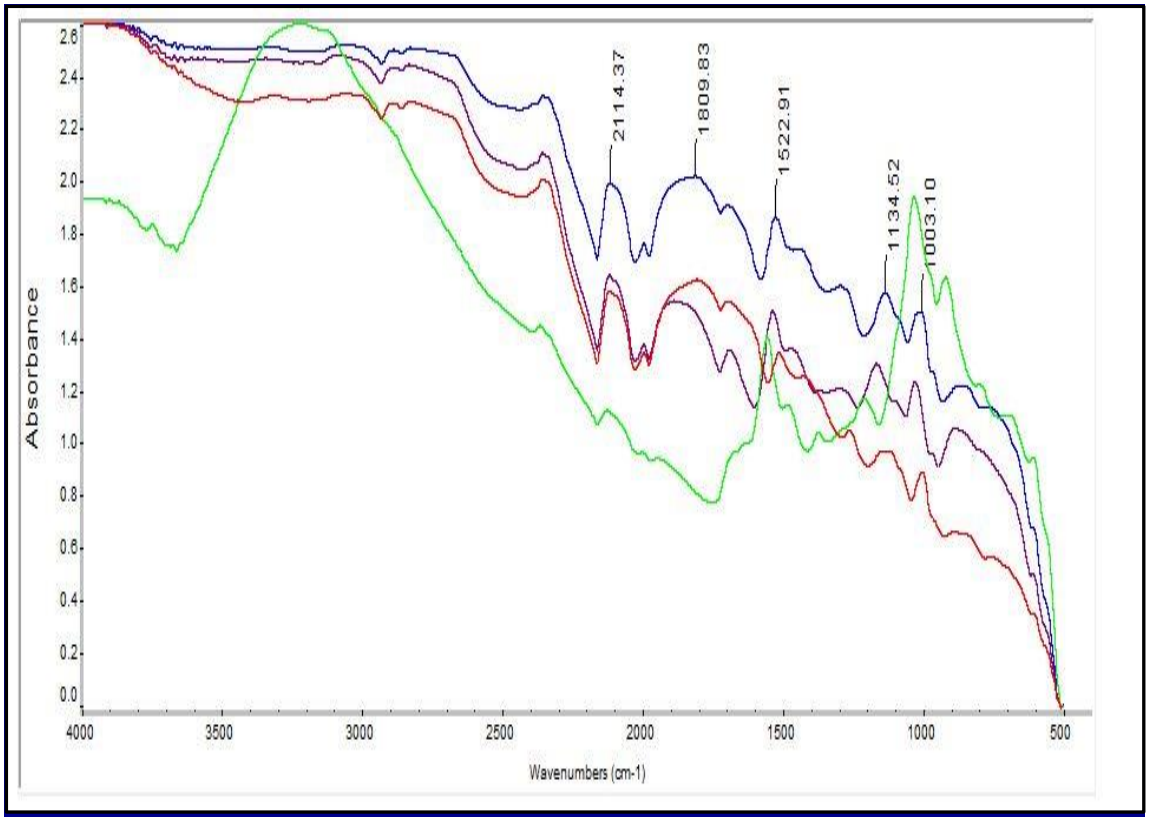


Figure 6.5. FTIR spectra of (——)PPy; PPy/FeCl3(——); PPy/ FeSO4(——); and PPy/APS (—) with the presence of Kolliphor P188

6.5.2 Morphological study of the synthesised PPy

SEM images of morphological feature studies of PPy synthesis are presented in Figure-6. These images clearly indicate that all the developed PPy powders were typically in tumour-like structure.

SEM images obtained at higher magnification power reveal that PPy powder was globular in shape. Sizes of these globules varied from formula to formula. A shown in Figure 6.6, when Ferric chloride was used, the size of PPy-$FeCl_3$ globules expanded remarkably to the range of 258 - 335 nm. Whereas, the use of $FeSo_4$ reduced the size of PPy-FeSo4 globules considerably, with sizes below 120 nm. In the case of APS, the size of resulting PPy-APS globules was the smallest among formulations and was approximately < 92 nm. Consequently all developed PPy in the presence of surfactant demonstrated nano-scale globular sizes which were smaller compared to the size of PPy powder prepared in reported research [250, 251]. Such findings are thought to promote the conductivity properties for

these PPy-formulations, which will be discussed comprehensively within the following sections.

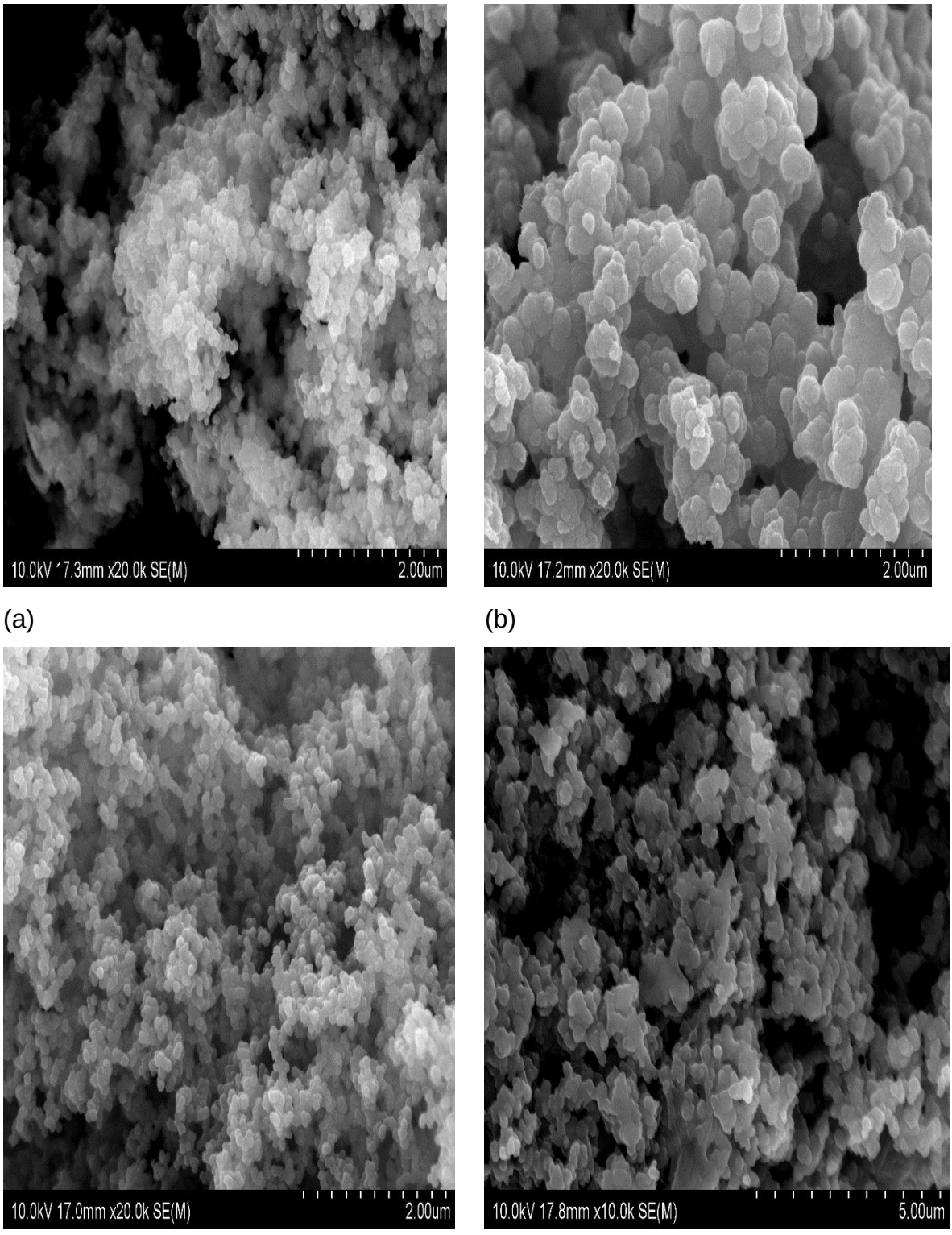


Figure 6.6. SEM images for various developed PPy formulations; (a) PPy-APS nanocomposite; (b) PPy-Anhydrous $FeCl_3$ nanocomposite; (c) PPy-$FeSO_4$ nanocomposite and (d) PPy- references

6.5.3 Electrical properties for the synthesised PPy

The electrical conductivity of PPy powder synthesis with different oxidizing agents and doped with a surfactant, was investigated following the well-known four-point probe resistivity measurement technique. This technique eliminates contact and wire resistance from measurements, and hence improves the accuracy of measurements [252, 253]. The current study was designed to investigate the electrical conductivity of developed PPy as a function of temperature; a protocol was developed investigating a temperature range between 20 to 40 °C [254].

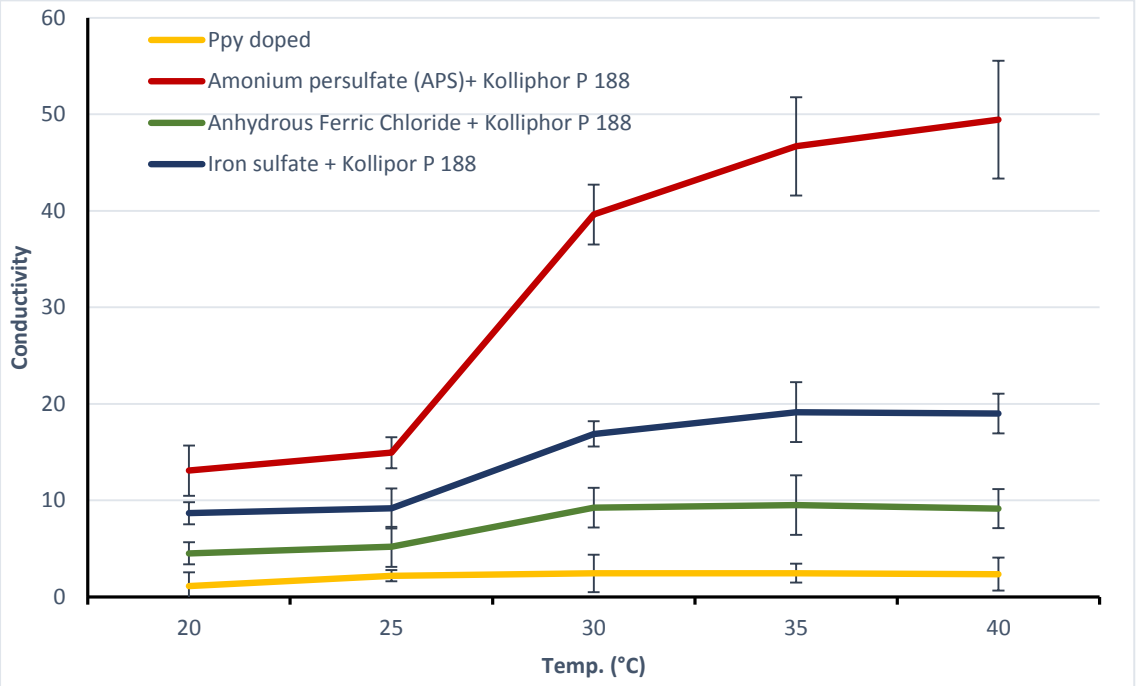


Figure 6.7 Electrical conductivity as a function to temperature for PPy powder of different developed formulations

Figure-6.7 shows that there is a direct linear relationship between the electrical conductivity of PPy disks and applied temperature. As per data, as temperature increases the electrical resistance decreases due to the efficient transfer of charge in the PPy ring [255-257], which in further developments may lead to considerable improvement in PPy conductivity. By demonstrating this conductivity dependence on temperature, PPy replicates the thermal behavior of metallic conductors. Another important finding was that the inclusion of surfactant as a dopant in the PPy polymerisation process improved the conductivity of these formulations remarkably. The highest improvement in conductivity was found in the PPy-APS doped surfactant formula, as it increased from 13 S/cm at ambient temperature to as

high as 49.4 S/cm at 40 °C. The lowest value was associated with PPy-$FeSO_4$, with a lower increase from 5.2 to 9.15 S/cm. Many factors may contribute to the differences within conductivity dependence temperature behavior, such as the molecular weight of oxidising agents, the charge of dopants, and most importantly, the efficacy of conjugation of dopants to the PPy [258] . This prominent improvement can be explained to the effect of dopants and thermal stress on microscopic and macroscopic conductivity of PPy. Basically, electrical conductivity is influenced by two factors. First, the microscopic conductivity depends on doping-related characterisation in terms of dopant type, dopant conjugation, and the length of the resulting polymer chain. Whilst the macroscopic conductivity depends on the crystalline form of resulting PPy powder and compactness [259]. In this study, utilising Kolliphor P188 as a dopant was performed efficiently, leading to synthesis of PPy particles having nano-scale sizes and highly ordered structures. In addition, the resulting particles can be easily compacted, and therefore enhanced macroscopic conductivity. As per morphological study findings, PPy particles were in nano-scale and existed in highly ordered globules. Therefore, it can be used to supports this explanation.

Another important parameter which is thought to contribute in the conductivity improvement is the thermal effect on the PPy chain orientation. It was found that increasing temperature leads to enhancing the conjugation length of the PPy chain, and allows rearrangement of molecules, thereby improving electron delocalisation. As a result, heating will improve microscopic conductivity. By improving the microscopic and macroscopic conductivity of PPy formulations, a significant improvement with conductivity has been observed in the developed PPy formulation. This finding from the study, in terms of conductivity, is regarded as an important step in PPy utility as it reports higher values in comparison with previous reports. Upon these findings, PPy-APS was selected for the following development and associated investigation studies.

6.5.4 Electrical conductivity study of Bioimprinted Polymeric implant

PPy has been applied in biomedical technology-related studies comprehensively. Characterisation as a highly conductive polymer possessing excellent biocompatibility makes it an excellent candidate for various applications in the biomedical arena. However, the adaption of PPy was hindered by compositional drawbacks, in terms of being brittle and nonbiodegradable. The study suggests that developing a bioimprinted polymeric scaffold may

represent an excellent alternative to overcome these issues. Three different polymeric materials were utilised to improve this substrate and were investigated to assess their electrical conductivity. Developing the PPy/APS bioimprinted implant was performed following the protocol described in the Methodology section. The concentration of PPy/APS was optimised to achieve a flat surface and a high conductivity at the same time, and hence to avoid a poor replication during bioimprinting process. High concentration of PPy/APS was found to have a rough surface which produced poor experimental replication. The conductivity of developed bioimprinted polymeric substrates was performed following the four-probe resistivity measurement technique, at room temperature. In this study three polymers of Chitosan, Gelatin and PDMS were used to build bioimprinted polymeric membrane. Polymers of chitosan and gelatin have been chosen due to their organic nature and having a long record of use in developed drug delivery systems. PDMS was selected due to its physical stability.

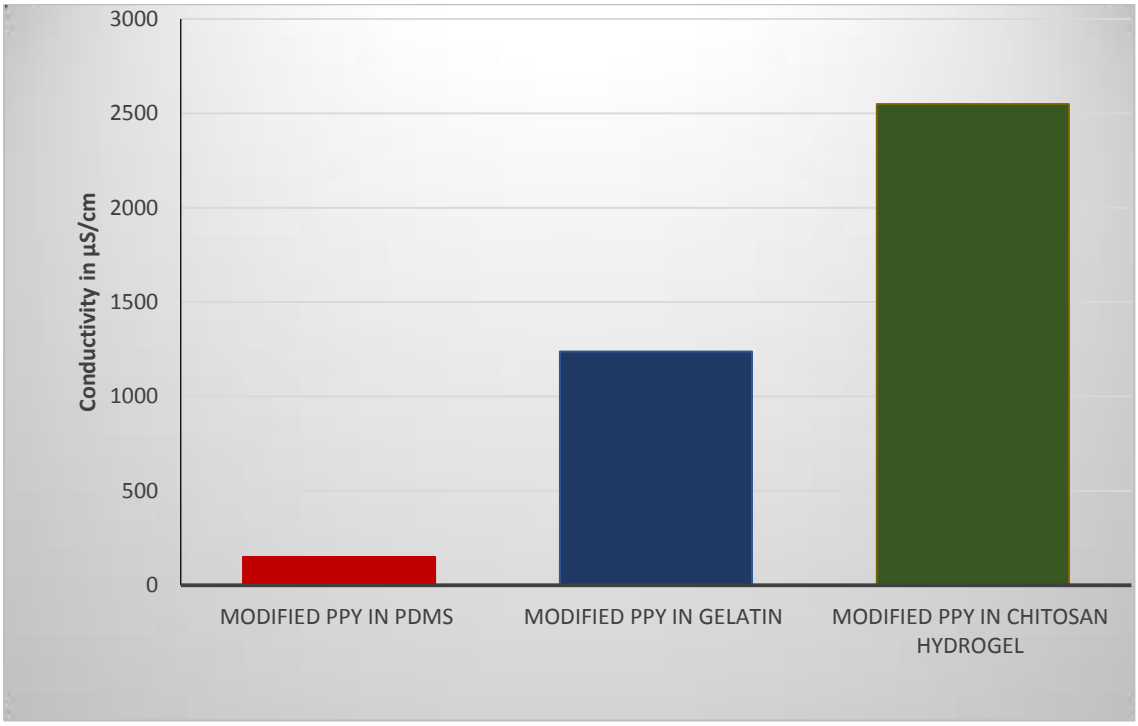


Figure 6.8. Electrical conductivity of PPy/APS casting in chitosan scaffold

Figure 6.8 shows the conductivity of these PPY/APS polymeric scaffolds at room temperature. Results confirmed that Chitosan/PPy-APS has the lowest resistance and hence the highest conductivity (2.5 S/cm) in comparison to other developed moulds. This can be

explained due to the effect of macroscopic parameters rather than microscopic. As all formulations used the same PPy-APS formulae, therefore microscopic effects will not be effective. During polymerisation, the chitosan will absorb more water content, whilst, the PPy-APS particles will retain water content within the structure of chitosan. This relatively high concentration of water within chitosan's structure will facilitate this improvement within electrical conductivity [260]. Similarly, but at a lower level, the conductivity of Gelation/PPY-APS mould can be explained based on water content. PDMS has demonstrated the lowest conductivity values, which can be explained due to the rigidity of mould.

This study has confirmed that the developed Chitosan/PPy/APS -chitosan polymeric implant has excellent electrical conductivity and it is higher than what has been reported by others [261], suggesting the efficacy and reliability of the developed protocols.

As per conductivity performance, the Chitosan/PPy/APS formulae was selected for further investigation.

6.5.5 Topographic study of Bioimprinted Polymeric Implant

SEM facilitated studies of the topography of the bioimprinted polymeric membrane developed using Chitosan/PPy-APS due to the availability within the facility.

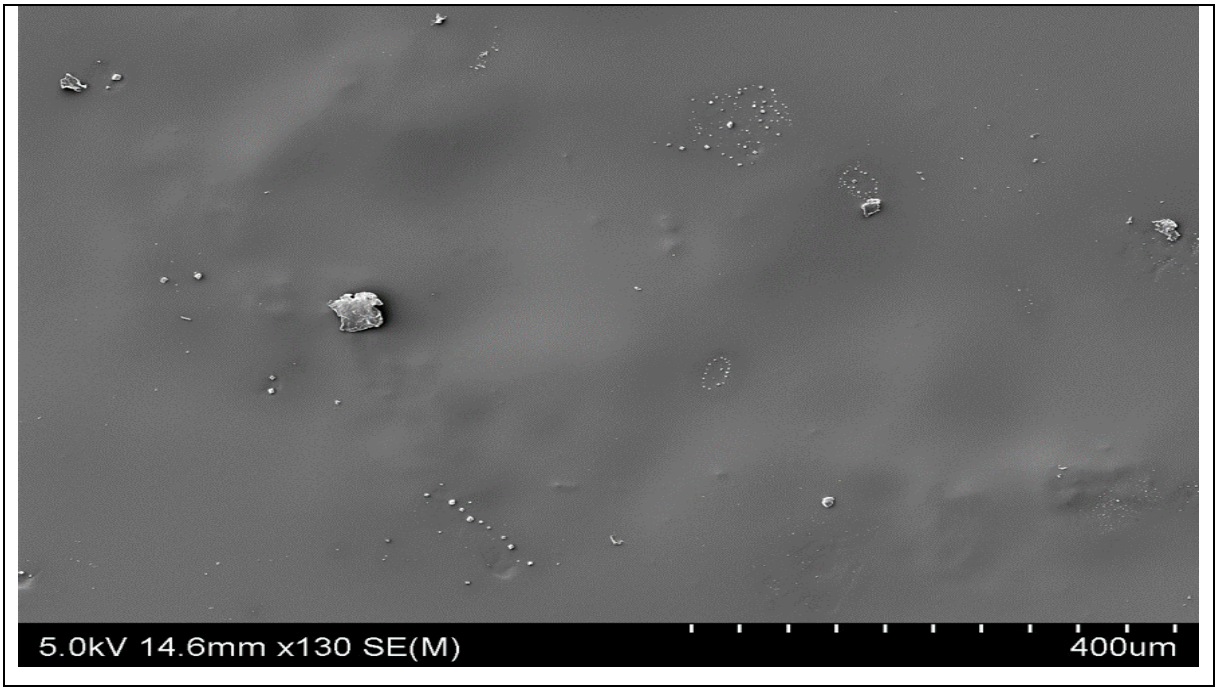


Figure 6.9 Topographical features for Bioimprinted polymeric membrane captured by SEM

By analysing SEM images depicted in Figure 6.9 of the Chitosan/PPy-APS membrane developed to replicate Caco-2 fixed cell features, it showed that the polymeric mould was able to replicate fine cellular features from the fixed cells. Replication of fixed cells can also be confirmed through the predominant protrusion on the polymer surface. Study findings suggest that replication of the fixed cells was optimal.

6.5.6 Bio-compatibility study of Bioimprinted implant

As per the study objective, the developed membrane is intended to be implanted within the hypoglossal vicinity, and therefore investigating the bio-affinity of this membrane is of paramount importance. An assessment was first performed to ensure the non-toxicity of this membrane to living cells. Prior to being used, the bioimprinted polymeric membrane was washed, UV-sterilized, and inserted into 5-cm plastic petri-dishs, cells were seeded at a density of 50,000 cells. cm^{-2} and incubated for a further 24 h to allow cells to attach the surface. Cells were then washed with PBS, fixed, and dried following the same steps mentioned earlier. Samples were then analysed using SEM.

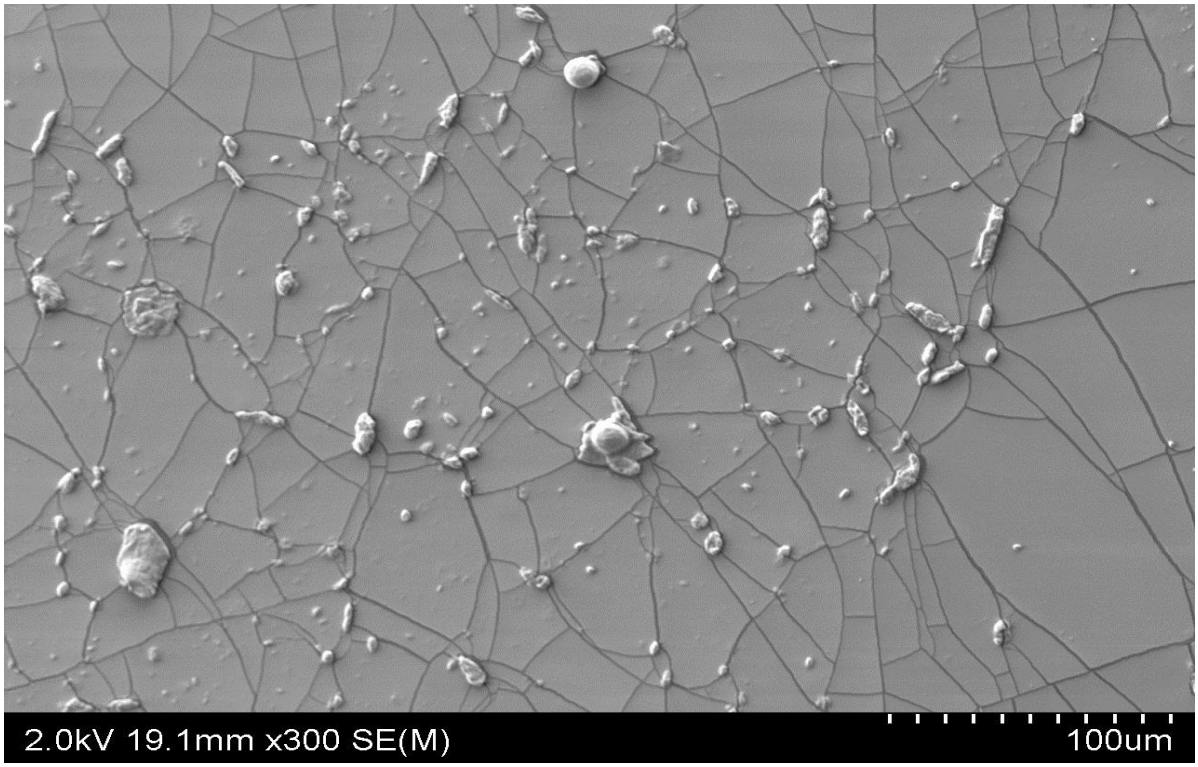


Figure 6.10 SEM images for Caco-2 cells attached to a bioimprinted polymeric membrane

As can be seen in Figure 6.10, SEM images clearly indicate Caco-2 cells were attached, and grew on the conductive membrane of chitosan/PPY-APS, suggesting that the membrane is highly tolerated by Caco-2 cells due to polymer's biocompatibility.

6.5.7 Investigate the Electrical stimulation on Bio-compatibility.

As per the study's aim, this developed bioimprinted polymeric membrane is intended to be implanted within the vicinity of the hypoglossal nerve to stimulate it electrically. Therefore, bio-affinity and tissue response towards electrical stimulation are of paramount importance to maintain the long-term efficacy of the implant. Previously, the biocompatibility of this proposed implant was confirmed using SEM images, and the tissue's response will be assessed during this part of this study, following a protocol developed for this purpose and using a flow cytometry technique.

Three DC values of 150, 600 and 1000 mV/mm were applied to the cells through the conductive polymeric substrate. Cells were assessed to evaluate the impact of these potential values. As per manufacturer's protocol, preparation using Annexin V kit processed samples to be evaluated for viable cells and to calculate the required amount of binding kit required for optimised cytometry. Preparation of samples that were subjected to higher values of DC potential (namely 600 and 1000 mV/mm), demonstrated that the number of viable cells decreased by 90 – 95 % of the cell density used in seeding. Therefore, samples from these potentials were unable to be analysed with flow cytometry.

The mechanism by which electrical stimulation at these higher DC magnitudes causes cells apoptosis can be explained based on the impact of such stimulation on the cell membrane. Cells maintain a quasi-electrostatic gradient between the intracellular and extracellular environments to perform their functions and ensure viability[262]. Applying DC potential is thought to alter the surficial membrane charge of the cell, and that may represent a major challenge to maintain cell's viability, eventually leading to induced cell death. Samples that were subjected to a DC potential of 150 mV/mm were analysed against the control samples and are presented in Figure 6.11.

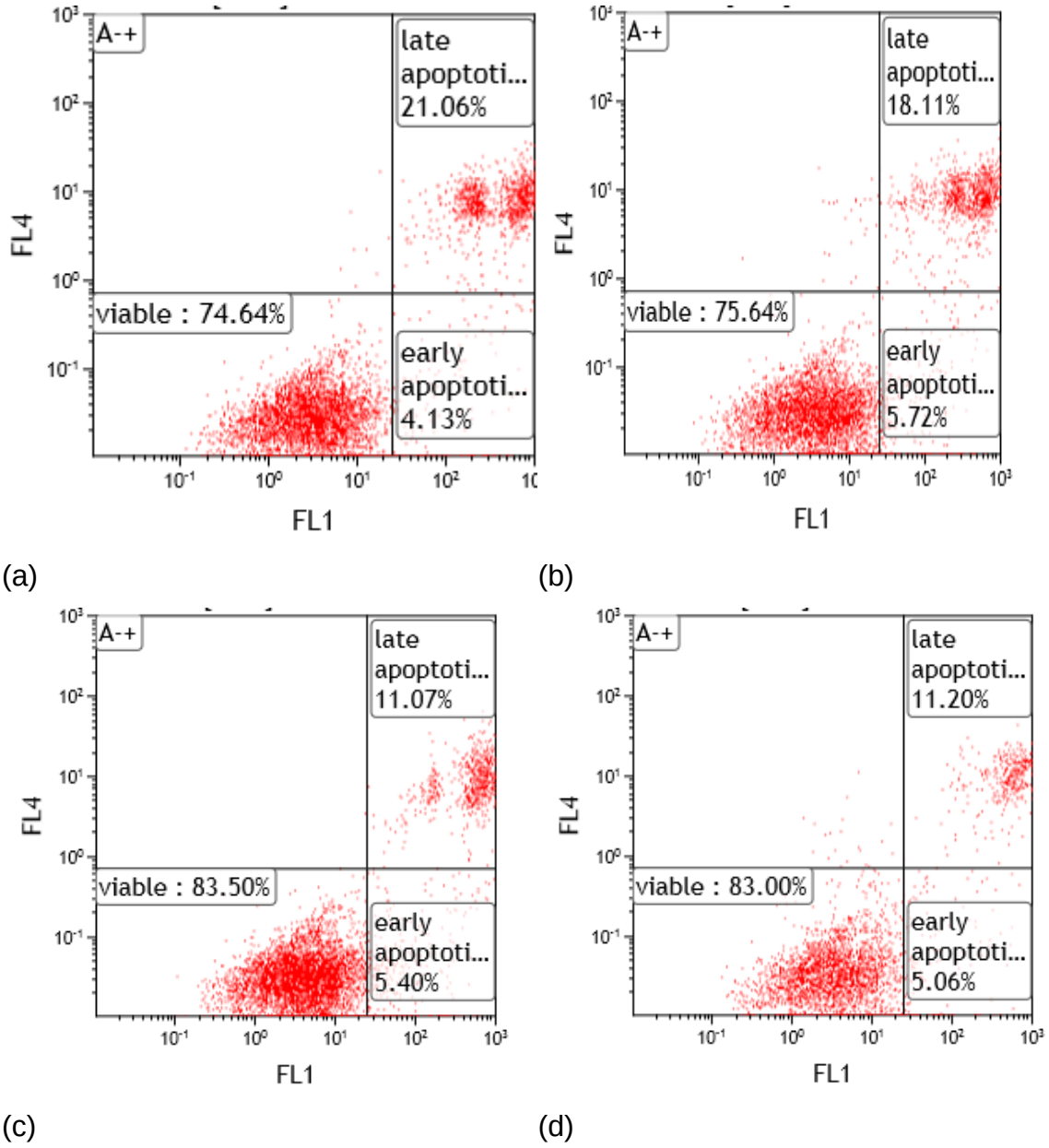


Figure 6.11. Tolerance of Caco-2 cells to the electrical stimulation of 150 mV/mm delivered through a conductive bioimprinted polymeric membrane (a,b) for reference where no electrical stimulation was applied, while (c,d) when electrical stimulation of 150 mV/mm was applied

As seen in the Figure 6.11, electrical stimulation at relatively low values was tolerated by attached cells as cells maintained their proliferative functions without been influenced by electrical stimulation. Results also reveal that applying electrical stimulation at relatively low values may promote cellular proliferation by 10.8%, reducing rates of apoptosis from 19.58 % to 11.13 %. Data confirms the positive impact of electrical stimulation using a lower value of DC on cell functions.

Findings from this study confirm the positive role of applying a relatively lower DC potential on cell proliferation. Also, the results indicate the harmful impact of applying higher DC magnitudes on cells. Results are in accordance with the findings reported by others [263].

6.6 Closure

Results presented in this chapter indicate that PPy synthesised with APS as an oxidant and Kolliphore P188 as dopant have a significantly smaller particle size within the nano-scale compared to other formulations tested. Electrical conductivity studies have confirmed that this nanocomposite PPy-APS has relatively low electrical resistivity and hence the highest electrical conductivity, therefore it was selected for further improvements. Nanocomposites were utilised to develop chitosan hydrogel-based implants to be used for electrical stimulation. *In vitro* studies have confirmed the biocompatibility of the implants' surfaces to attached cells in culture settings. Cells' tolerance towards the implants were detected using SEM techniques. Moreover, cellular tolerance towards applying electrical stimulation through implants was investigated following *in vitro* techniques. Results of flow cytometry have confirmed that cells tolerate up to 150 mV/mm, and applications above this threshold affect cell viability severely. The results are furthered discuss in the following chapter.

Chapter 7
Discussion

7.1 Introduction

In previous chapters, this study investigated how fluid depletion affects upper airways following *ex vivo* and *in vivo* techniques, and results obtained from the characterisations were presented. This chapter discusses results obtained from both applied techniques and compares them between normal breathing conditions and application of CPAP. The impact of the PO superimposed on CPAP is further analysed and compared with results of previous breathing conditions. Finally, a recommendation for future work is presented prior to the thesis conclusion.

7.2 Effect of the CPAP (*ex-vivo/in-vivo* correlation).

Tracheal WF, presented in Chapter 4, corresponds to tracheal fluid depletion values that affect tracheal tissues. These conclusions are derived from procedures outlined in the methodology of Chapter-3. Fluid-depletion WF values resulting from applying CPAP may cause dryness within tracheal tissue, as quantified in Chapter 4. Consequently. there is alteration in tracheal efficacy in reconditioning elevated air flow rates supplied by CPAP. The tracheal WF values obtained in *ex vivo* conditions are compared to salivary flow rate values obtained from in vivo studies following spitting techniques using protocols and techniques described in Chapter 5. In order to be comparable, results obtained in Chapter 3, 4 and Chapter 5 are furthered analysed to calculate tracheal fluid-depletion per square cm since dryness affects the upper airways in square cm. Normalisation steps are as follows:

For the *ex vivo* results, the surface area of the trachea was calculated after completion of the experiment and the average of the total surface area exposed to air was 480 cm^2. By taking into consideration the average WF of 1.04 mL/min, the tracheal water flux per sq.cm of the is equal to 2.17 $\mu L/cm^2$.min. Therefore, the total WF that contributed from ASL and been used to reconditioning the inhaled air under normal breathing condition for one hr is equals to 130.24 $\mu L/cm^2$ hr. This number refers to tracheal capacity per hour. However, applying

CPAP at different operating pressures altered tracheal efficacy to recondition pressurised air supplied by CPAP. Achieved results are presented in Table 7.1.

Table 7.1. Tracheal capacity at different experimental conditions

CPAP operating pressure (cmH2O)	**Average WF per min.(mL/min)**	**Tracheal water flux (µL/min.cm^2)**	**Tracheal Capacity (µL/cm^2.h)**
Normal breathing conditions	1.04	2.17	130.2
@ 5	0.54	1.125	67.5
@ 10	0.30	0.625	37.5
@ 20	0.25	0.521	31.25

For *in vivo* studies, the salivary flow rate is considered as an indicator of dryness attributed to applying CPAP on patients with the OSA. To compare these set of results with those obtained from *ex vivo* studies, a normalisation was implemented to ex-vivo results obtained when 10 cmH_2O pressure was applied. The in vivo study was performed at only 10 cmH_2O, to avoid any health damage of participants. Following from the previous discussion, applying this value of CPAP affected the salivary flow rate to range from 0.42 to 1.23 mL/min. Assuming that the oral cavity of adults has an average surface area of 214.7 ± 12.9 cm^2, the salivary flow rate can be presented as 1.5 to 5.7 µL/cm^2 (Table 7.2) [264].

Table 7.2. Total salivary flow rate of participants with CPAP.

Patients	BMI kg/m2	Total salivary flow rate µL/min.cm^2	% reduction in salivary flow rate with the CPAP
8	28.3	3.08	51.86
6	30	1.97	69.15
3	30.7	2.55	60.19
1	31.7	2.31	63.85
16	31.8	2.88	54.96
7	32.3	3.76	41.31
10	34.4	3.92	38.78
17	35.2	5.73	10.49
2	35.9	1.95	69.57
12	37.2	3.17	50.48
14	38.1	5.73	10.41
11	49.1	4.35	32.05

Comparing results obtained from both *ex vivo* and *in vivo* studies highlights various points:

- According to *ex-vivo* results, applying CPAP at 10 cmH_2O the tracheal capacity was altered to reconditioning processed air by a percentage up to 71.2 % of their capacity at normal breathing conditions.
- The effect of applying CPAP on deterioration of the upper airway has been confirmed. As per *in-vivo* studies, CPAP at 10 cmH_2O affected the salivary flow rate of participants significantly. Reduction percentages were found to vary among individuals. However, patients with a BMI of $\leq$ 32.3 kg/m^2 suffered from severe saliva reduction percentages between 41.3 to 69.1 %.
- *Ex-vivo*/ *in-vivo* correlations can be found between patients with relatively lower and medium BMI values.

This study suggests that there is a high correlation between the ex-vivo and in vivo results, and the depth of saliva or mucous lining the cellular surface has similar depth. As per tissue structure only the periciliary liquid layer (PCL) can be used to re-conditioning of supplied air [265]. It has been reported that PCL depth is approximately 7 µm and it governs the capacity of the ASL [210]. Therefore, these tissues demonstrate similar reaction towards elevated

pressure. To our knowledge, such findings are highlighted for the first time within such an excellent correlation. Results are in accordance with previously reported findings ([142, 144, 208, 266]. The results may indicate the efficacy of the experimental setup and the reliability of the findings.

7.3 Effect of PO (*ex-vivo/in-vivo* correlation).

Similarly, the impact of applying PO superimposed on CPAP as a means of improving dryness condition and fluid depletion is investigated following both *ex vivo* and *in vivo* techniques. As per Table 7.3, applying PO at different frequencies has improved tracheal WF significantly, with the highest improvement of 78.8% observed at a higher frequency of 30 Hz. The lowest improvement was recorded at 5 Hz, suggesting a linear relationship between the applied frequency of PO and improvement within the tracheal WF values. It also seems that applying PO restores fluid-depletion within tracheal tissues initially caused by applying CPAP alone.

Table.7.3. Tracheal Capacity at the CPAP and PO at different experimental frequencies

Time (min.)	**%Increase in WF (mL) at CPAP of 5 cmH_2O**	**%Increase in WF (mL) at a PO of 1 cmH_2O and Frequency of**		
		5 Hz	**20 Hz**	**30 Hz**
5	-38.4	7. 6	15.7	33.4
15	- 40.5	12.0	25.2	45.9
30	- 43.1	35.3	47.2	58.1
60	- 45.5	45.4	66.2	68.6
90	- 48.2	54.9	72.1	78.8

The aforementioned tracheal WF values obtained from *ex vivo* studies are further analysed to calculate tracheal capacity of reconditioning air supplied by CPAP and PO, per surface area,

following the same steps described in section -7.2. Results are presented alongside other tracheal WF for comparison.

Table 7.4 shows that applied PO at 5 Hz has improved tracheal WF up to 1.75 µL/cm^2. This represents a 55% increase within WF compared to results obtained by applying only CPAP. This improvement in tracheal WF becomes even more so when applying higher frequency, up to 78%.

Table 7.4. Tracheal capacity per surface area at different experimental conditions.

CPAP of 5 cmH2O and PO at frequencies of	**Average WF per min.**	**Tracheal water flux µL/cm^2**	**Tracheal Capacity (µL/cm^2.h)**
Normal breathing conditions	1.04	2.17	130.2
@ 5 cmH_2O	0.54	1.125	67.5
Plus, PO@ 5 Hz	0.84	1.75	105
Plus, PO@ 20 Hz	0.93	1.94	116.4
Plus, PO@ 30 Hz	0.96	2.0	120

In order to establish *ex vivo* / *in vivo* correlations, results of salivary flow rates were obtained from participants of the clinical trial following procedures and protocols outlined in Chapter-5. Values were further analysed to calculate the salivary flow rate in square cm. The calculation is performed following the same procedure described in the Section-7.2. Results along with their improvement percentages are presented in Table 7.5. Major observations can be revealed from the table. First, by applying PO in conjunction with CPAP on OSA participants for full 8 hours treatment sessions there is significant improvement in their total salivary flow rate. Improvement percentage values varied among individuals from 6.8 % to

129 %. No correlation between BMI of participants and improvement percentages of their salivary flow rates was found.

Table 7.5. Total salivary flow rate of participants with PO superimposed on CPAP.

Patients	**BMI kg/m2**	**Total salivary flow rate after the PO µL/min.cm^2**	**% Improvement in salivary flow rate with the PO**
8	27.6	3.2	49.6
3	30.7	2.3	26.4
16	31.8	5.44	6.8
7	32.3	3.94	21.8
17	35.2	4.54	22.0
2	35.9	2.1	27.9
14	38.1	5.1	34.2
11	49.1	2.5	9.9

By comparing *ex vivo* results of tracheal WF per surface area with those of salivary flow rate of participants, study findings are also highlighted:

- applying PO superimposed on CPAP following *ex vivo* techniques improves humidification quality of processed air by enhancing tracheal WF values. Such enhancement percentages vary from 55 to 78 % depending on the applied frequency. The higher frequency produces a higher improvement within tracheal WF values.
- Applying the same condition of PO and CPAP on participants with OSA has improved their salivary flow rate by 6.8 to 49.6 %.
- 36% of participants produced a high accuracy of *ex vivo/ in vivo* correlations. Unlike findings of the previous section, the result suggests that improvement induced by PO are variable among individuals. There are many reported inconsistencies in the response of patients towards PO conditions [267-269].
- Improvement within tracheal WF is obtained due to the well-known " muscle-relaxant" effect induced by applying PO waves [270, 271].
- However, the mechanism by which improvement within salivary flow rate occurs is likely due to neural stimulation of main secretory glands, as explained in Chapter-5.

7.4 Effect of applying the CPAP vs applying PO

The effect of CPAP on the upper airway specifically alters the humidification quality of air. Results of these studies have confirmed the occurrence of dryness within the upper airway, and such dryness leads to poor compliance in patients with OSA. However, quantification of fluid-depletion affecting upper airway tissues has not been reported. In addition, the impact of applying PO superimposed on CPAP as a way to diminish dryness has not been investigated comprehensively.

Comparison between CPAP and PO treatments has been performed in this study and findings are indicative of improvements with applications:

- Both *ex vivo* and *in vitro* studies have confirmed the impact of CPAP on tracheal tissue and oral cavity tissues leading in both situations to dryness. Air reconditioning capacity of these parts is eventually altered. Reduction percentages in individuals vary.
- Imposing PO on CPAP improved both tracheal WF and also salivary flow rate among individuals.
- *Ex vivo/in vivo* correlation of applying CPAP is more profound, more accurate, and more consistent than other findings of applying PO.

7.5 Electrical stimulation treatment

The results presented in Chapter 6 correspond to the characterisation of the implant developed following procedures and resources described in the same chapter reveal various outcomes:

- Polypyrrole has been successfully synthesised in the presence of Kolliphor P188, acting both as dopant and surfactant. Identification tests have confirmed the success of developed chemical polymerisations setup to produce PPy modified with APS and Kolliphor P188. Morphological studies using SEM technique have indicated the sub-nano sizes of PPy particles. Furthermore, the developed implant has shown its biocompatibility through in vitro techniques.

- Most importantly, the implant was electrically-conductive, and results obtained from the test confirmed that both PPy/APS/ Kolliphor P188 nanocomposites and implants are electrically-conductive.
- Biocompatibility of implant surfaces was tested, and results obtained from corresponding tests have confirmed its non-toxicity. Furthermore, applying electrical stimulation to the attached cells has confirmed that a signal lower than 150 mV/mm can be tolerated by the attached cell population.

7.6 Conclusions

The objectives of this thesis were designed to investigate the effect of CPAP on humidification quality of air in the upper airway, and to quantify it, experimentally following *ex vivo* and *in vivo* techniques. Investigation of the effect of superimposing PO on improving humidification quality of air affected by CPAP was also quantified experimentally following *ex vivo* and *in vivo* techniques. Furthermore, development of polymeric medium to act as an electrode for delivering electrical stimulation as an alternative to CPAP, assessing its morphological surface, electrical conductivity, biocompatibility, and to quantify its biocompatibility while applying electrical stimulation, followed *in vitro* techniques. These objectives, in addition to comprehensive literature review regarding OSA and current treatments were described in Chapter-2. In chapter -3, protocols and resources, and ex vivo experimental setups utilised to calculate humidification parameters were described. Chapter 4 presented all *ex vivo* results of applying different breathing conditions (via CPAP and PO) on normal breathing patterns and analysing them to calculate TWC of the processed air and tracheal WF. Chapter-5 was designed to described protocols required to conduct a clinical trial. Procedures were followed to collect saliva, and results were followed by a brief discussion. Chapter-6 presented lab procedures and protocols to develop a bioimprinted conductive polymeric electrode, and results of its characterisation and evaluation of biocompatibility were presented. Finally, chapter 7 presented a comprehensive discussion for the results obtained in chapters 4, 5, and 6.

By comparing the study's' findings with their objectives, we found that:

1 Humidification parameters of the upper airway, in terms of TWC of the processed air and tracheal WF at various breathing conditions, were thoroughly investigated. Study

findings suggest that there is a forward relationship between CPAP operating pressures and values of tracheal fluid-depletion (WF).

2 Dryness associated with applying CPAP has been investigated and quantified following in vivo techniques, and the outcome of these studies shows that all OSA patients tested, who are a regular user of the CPAP, have relatively low salivary flow rates. Furthermore, applying a full treatment session of CPAP deteriorated their salivary flow rate. This outcome supports the ex vivo study's findings.

3 The effect of applying PO superimposed on CPAP improves dryness associated with the CPAP. This was investigated following *ex vivo* and *in vivo* studies. Findings have confirmed the direct linear relationship between the frequency of the PO and improvement percentages, in particular within low to a medium operating pressure of the CPAP.

4 In vivo studies and outcomes are fully supporting results obtained from ex vivo studies.

5 Developing a novel bioimprinted conductive polymeric implant was found to efficiently address the poor long-term performance of the current electrical stimulation devices. The developed implant was able to demonstrate biocompatibility of its surfaces with surrounding tissues. Eventually, this technique can address dryness and other drawbacks associated with the CPAP.

7.7 Future Work

The current findings of this project can be furthered improved within the following areas:

1 Conduct a new phase of this project through developing new non-invasive techniques to replace or improve the current CPAP, using chemical stimulation, to address dryness associated with CPAP.

2 Conduct a new phase of this project through developing new non-invasive techniques to replace or improve the current CPAP, using mechanical stimulation, to address dryness associated with CPAP

3 Both proposals need first to develop device concept and build it to convoy either chemical or mechanical stimulation.

4 These types of stimulation may also work alongside the CPAP, through developing the CPAP to provide pressurised air and deliver stimulation to improve dryness.

5 Feasibility and then efficacy of these proposal techniques need first to be investigated following ex vivo technique.

6 It may also require investigating their performance by applying proposed techniques to animal models such as guinea pigs or rabbits.

7 Performing in vivo testing by recruiting participants to test these proposed techniques.

8 Further investigation of electrical stimulation can be performed to assess the biocompatibility of implants using different cells line and different techniques.

9 Further improvement to the electrical stimulation can be suggested by loading it with drugs that can enhance muscle or neural performance.

10 Testing of more frequencies and amplitudes of PO may also worth investigating for their reliability and feasibility.

Chapter 8
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