Treatment of glue ear biofilms using a combination of N-acetylcysteine and antibiotics in a modified release form

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Abstract

Aims

There is increasing evidence that biofilms are implicated in otitis media with effusion. The projects aim was to test the in-vitro efficacy of clindamycin and rifampicin with N-acetylcysteine against middle ear biofilm and elucidate the mechanism of action of N-acetylcysteine on extra-cellular slime, major constituent of biofilm. Hypothesis investigated: if N-acetylcysteine breaks down extra-cellular slime, this might enhance antibiotic eradication of bacteria by removing the protective effects of a biofilm state.

Methods

N-acetylcysteine with and without rifampicin / clindamycin combination was tested in vitro against biofilm of *Staphylococcus aureus* (F2315), previously isolated from glue ear fluid. Biofilms were grown on silicone discs and separately on pegs in 96 well microtitre plates - Minimum Biofilm Eradication Concentration (MBEC) assay. The effect of N-acetylcysteine on extra-cellular slime of F2315 was examined using a glycosaminoglycan alcian-blue assay and cellulose acetate electrophoresis.

Results

Biofilms on silicone discs were eradicated by N-acetylcysteine (50mg/mL) and rifampicin / clindamycin combination 100 times above the minimum inhibitory concentration (MIC) after 24hrs. This was largely due to low pH of N-acetylcysteine. Subsequent neutralisation in the MBEC assay showed eradication of 4 day-old biofilms with N-acetylcysteine (5mg/mL) and rifampicin / clindamycin combination (100MIC) after one-week. This was not possible with N-acetylcysteine alone or antibiotics (rifampicin / clindamycin) alone at 100, 1000, or 10000 MIC after one-week. Non–pH neutralised N-acetylcysteine caused precipitation of extra-cellular slime that reversed on neutralisation. Neutralised N-acetylcysteine did not affect extra-cellular slime quantity. Addition of neutral N-acetylcysteine to extra-cellular slime changed the electrophoresis pattern, reducing stain density with disappearance of a halo.

Conclusion

Combining N-acetylcysteine with rifampicin and clindamycin seems effective in eradicating biofilms. N-acetylcysteine may affect the molecular structure of extra-cellular slime. The use of N-acetylcysteine with rifampicin and clindamycin as therapeutic agents delivered to the middle ear using a biodegradable polymer is being explored.

List of abbreviations

ABR	Auditory brainstem response
AOM	Acute otitis media
API®	Analytical profile index
ATP	Adenosine triphosphate
BRIG	Biomaterials related infection group
CA	Cellulose acetate
cGMP	Cyclic guanosine monophosphate
Clind	Clindamycin
CLSM	Confocal laser scanning microscopy
CoNS	Coagulase-negative *staphylococci*
CRS	Chronic rhinosinusitis
CS	Chondroitin sulphate
CSOM	Chronic suppurative otitis media
CSF	Cerebrospinal fluid
CFU	Colony-forming unit
dB	Decibel
dBHL	Decibel hearing level
dH_2O	De-ionised water
DNA	Deoxyribonucleic acid
ECS	Extra cellular slime
EPS	Extra-cellular polymeric substance
EVD	External ventricular drainage
GAG	Glycosaminoglycan
GTP	Guanosine triphosphate
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-8	Interleukin-8
IL1-β	Interleukin-1 beta
ISA	Iso-sensitest agar
LPS	Lipopolysaccharide

MBC	Minimum bactericidal concentration
MBEC	Minimum biofiom eradication concentration
MEE	Middle ear effusion
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
mRNA	messenger Ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MRSE	Methicillin-resistant *Staphylococcus epidermidis*
MSSE	Methicillin-sensitive *Staphylococcus epidermidis*
NAC	N-acetylcysteine
NaOH	Sodium hydroxide
NaN_3	Sodium azide
NICE	National institute for health and care excellence
NO	Nitric oxide
OD	Optical denstiy
OME	Otitis media with effusion
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIA	Polysaccharide Intercellular Adhesin
PLGA	Poly-lactic-co-glycolic acid
RAOM	Recurrent acute otitis media
Rif	Rifampicin
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscope
SCV	Small colony variant
TARGET	Trial of Alternative Regimens in Glue Ear Treatment
TNF-α	Tumour necrosis factor alpha
TSA	Tryptone Soy Agar
TSB	Trypticase Soy Broth
VRE	Vancomycin-resistant *enterococcus*

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Section 1. Introduction

1.1 Otitis media with effusion (OME) and clinical implications

Humans are an intelligent species owing to their high cognitive skills and the ability to communicate with each other on a complex level. This communication forms a central role in facilitating our everyday interactions with others and our surroundings. Communication can be either verbal or non-verbal, and is diverse depending on cultural and social demographics. Although the majority of our communication is non-verbal through gestures and body language (Hess & Thibault, 2009), oral communication through speech is important for conveying informational content. Verbal communication depends on the production of sounds of varying quality and frequencies by the sender and the ability to detect those sounds by the receiver.

Although hearing is not as important for survival in modern day humans, it certainly plays a big role in social interactions, recreational activities, and for daily communication. The loss of hearing can have major psychological impact in previously normal hearing people. In children hearing is central for developing speech and facilitating learning in early pre-school years.

Otitis media with effusion (OME), more commonly known as glue ear, is the main cause of hearing loss in children in the developed world. Prolonged hearing loss secondary to OME may have linguistic, developmental, behavioural, and social consequences (Butler & Macmillan, 2001, Paradise *et al*., 2000). Parents of children with hearing loss have high levels of anxiety (Bellussi *et al*., 2005), and this may be secondary to guilt feelings that their children are 'missing out' in this developmentally critical period of their lives. In order to understand the importance of OME and the difficulties in its treatment, the normal and abnormal physiologies of the middle ear must be set out.

1.1.1 Middle ear in health and disease

The human ear consists of the outer ear, middle ear, and inner ear. The external auditory canal of the outer ear is a tube that funnels sound towards the tympanic membrane and into the middle ear. The bony ossicles in the middle ear connect the tympanic membrane to the oval window of the inner ear. The bony ossicles are arranged in such a way so as to amplify the sound vibrations that reach the tympanic membrane and transmit those into the inner ear vestibulo-cochlear apparatus through the oval window. The vibrations inside the cochlea oscillate the fluid in the cochlea that in turn moves the inner hair cells attached to the basilar membrane. Those cells are responsible for changing mechanical vibrations to electrical signals depending on the amplitude and frequency of the vibrations. The neural signal is transmitted via the cochlear nerve to the brainstem where they pass through the superior olivary complex and the inferior colliculus (Figure 1.1). From there the signals travel to the medial geniculate nucleus before ending in the auditory cortex of the temporal lobe so that the perception of sound is experienced. The integrity of this pathway can be tested using auditory brainstem response (ABR). This involves placing electrodes on the scalp to measure electrical activity typically generating five waves, each corresponding to an anatomical region of the auditory pathway (Moiler, 2007).

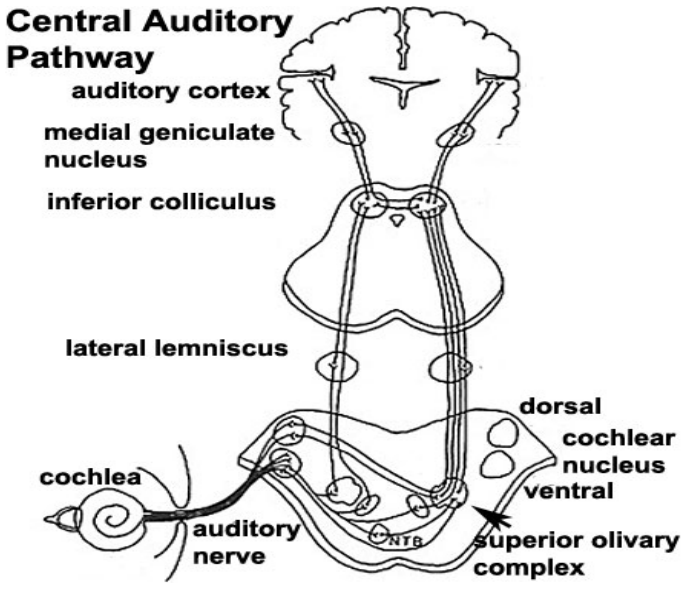


Figure 1.1 Central auditory pathway showing the neural signal route from the cochlea to the auditory cortex.
http://php.med.unsw.edu.au/embryology/index.php?title=2011_Lab_10_-_Feta

The normal functionality described is disrupted in OME by a build-up of fluid ("glue") inside the middle ear. This fluid may be either serous or viscous depending on the chronicity of the OME and points to differences in composition that is alluded to in Section 1.2.1. The fluid interferes with the normal conduction of sound as the tympanic membrane and ossicles are not able to vibrate freely. The resulting effect is a conductive hearing loss, typically around 25 to 40 decibels (dB) in the affected ear. Children present with hearing impairment and speech problems, which is usually picked up by parents who notice a change in the child's behaviour, performance at school, or language development. In contrast to acute otitis media, children do not have acute ear pain, fever, or malaise (Williamson, 2011).

1.1.2 Prevalence, risk factors, and natural history

OME is common in children, and 80% of pre-school children will develop OME at least once by the age of 4 years, peaks of incidence being found at the age of two years and five years (Zielhuis *et al.*, 1990). A study in the United States showed a cumulative incidence of OME for pre-schoolers to be 53% in the first year, and 61% in the second year (Casselbrant *et al.*, 1985). A large epidemiological study performed in Pittsburgh involving 2253 infants in the first two years of their lives (Paradise *et al.*, 1997) showed that 91.1% of infants developed middle ear effusion in the first two years of life, and out of those 52.5% had bilateral effusions. The paper did not differentiate between effusions secondary to acute otitis media (AOM) and OME, although the two are interlinked. Patients who have an episode of AOM are more likely to develop OME and vice versa (Martines *et al*., 2011).

In an attempt to understand OME, epidemiological studies have identified various risk factors, some factors having stronger correlation than others. Children with atopy such as eczema or asthma, have a 12 times higher chance of having OME than children without atopy (Martines *et al*., 2011). Illiterate mothers are twice as likely to have a child with OME as mothers of other education levels. The reasons for this are not entirely clear but may reflect less maternal knowledge about the risk factors for OME such as smoking and bottle-feeding. Children with a previous upper

respiratory tract infection or AOM episode are three times more likely to develop OME. Children who snore are four times more likely to develop OME. This is likely due to enlarged adenoids that may interfere with eustachian tube function by direct effect of adenoid size or through providing a bacterial pool in the nasopharynx that predisposes to upper respiratory infections and otitis media. Other risk factors identified include day care attendance, large number of siblings, low socio-economic group, bottle-feeding, and household smoking (Casselbrant *et al*., 1985, Teele *et al*., 1989).

Although OME is very common, spontaneous resolution occurs in the majority; half of cases between the ages of two and four resolve within three months, and about 95% resolve within a year, but 5% persist and become chronic (Zielhuis *et al*., 1990). In addition, not all children with OME will have a significant hearing loss, and the degree of disability will depend on the amount of drop in hearing. This has implications on management of the condition and guidelines have been developed in account of this fact. In the UK, current practice involves offering intervention only if there is an observed hearing loss of 25 to 30 dB in the better ear after a watchful waiting period of 3-months to avoid over - treating children (ENT UK 2009).

1.1.3 Clinical sequelae

In order to fully appreciate the impact of OME, it is important to look at the various outcomes caused by the disease. These can be broadly categorised into audiological sequelae of hearing loss, speech, language and learning delay and otopathological sequelae of the middle ear. One of the most important outcomes of OME is the degree of hearing impairment as this is closely related to the development of a child. There are various degrees of hearing loss with the equivalent level of impairment showed in table 1.1 (WHO 1991).

Degree of hearing loss	Decibel hearing level (dBHL)	Impairment level
Normal	25dBHL or less (better ear)	No or very slight hearing problems. Able to hear whispers
Mild	26 – 40 dBHL (better ear)	Able to hear or repeat words spoken in normal voice at 1 meter
Moderate	41 – 60 dBHL (better ear)	Able to hear and repeat loud voice at 1 meter
Severe	61 – 80 dBHL (better ear)	Able to hear and repeat some words when shouted into better ear
Profound	81 dBHL or louder (better ear)	Unable to hear or understand even a shouted voice

Table 1.1 World Health Organisation (WHO) classification of various degrees of hearing loss and equivalent impairment

Hearing loss secondary to OME is usually between mild to moderate but some children will have near to normal hearing. An early study that examined the hearing acuity of children with OME showed an average air conduction level of 27 dBHL at frequencies 500 Hz, 1000Hz, and 4000Hz. Fifty percent of ears had hearing thresholds poorer than 23 dBHL, and twenty percent were poorer than 35dBHL (Fria *et al*., 1985). This illustrates the importance of performing a hearing test to quantify the level of disability prior to deciding on the need for intervention thus targeting the group of patients mostly affected.

Though there are multiple studies examining the impact of OME on speech and language development, some of the results are difficult to interpret due to the wide variability of methodology and lack of strict criteria to define outcome measures. Two meta-analyses of prospective studies have come to similar findings. OME was

found to account for a small negative association on receptive and expressive language (Roberts *et al*., 2004, Casby, 2001). Although the effects were small, most of the trials on OME excluded children who were 'at risk' with other co-morbidities such as Down syndrome, cleft palate, or craniofacial anomalies where it is known that OME is less likely to resolve spontaneously. Those 'at risk' groups often have other learning impairments, and a reduction in hearing is potentially more detrimental to their overall speech and language development (Whiteman *et al*., 1986). The other caveat with the studies in the meta-analysis is that they included children with a positive history of OME based on presence of middle ear effusion. A more relevant inclusion criterion is the presence of OME and a conductive hearing loss, in addition to the extent and duration of hearing loss (Casby, 2001).

For the reasons stated, the American Academy of Pediatrics Subcommittee on Otitis Media in 2004 issued recommendations advising clinicians to consider treatment for children more at risk of the detrimental effects of hearing loss, as well as to consider treating children who have risk factors that decrease the spontaneous resolution rate such as hearing loss of 30 dBHL or worse in the better ear, persistent effusion, and a history of previous tympanotomy tube.

Chronic untreated OME causes irreversible structural changes through mechanisms that are outlined below. The eardrum consists of three layers, an outermost skin layer of squamous epithelium, an innermost modified respiratory epithelium, and a middle lamina propria layer consisting of fibrillar collagen. The collagen layer is important for the structural integrity of the eardrum, and damage to this causes atrophy predisposing to otopathological sequelae such as atelectasis, adhesive otitis media and cholesteatoma (Jennings *et al*., 2001). Matrix metalloproteinases (MMPs) are thought to play a key role in the degradation of the collagen and are found in higher concentrations in children with chronic OME who have undergone multiple ventilation tube insertions. Thick effusions have a higher concentration of interleukins and MMPs compared to thin serous effusions implying that the chronicity of OME and the visco-elastic properties of the middle ear effusion are important factors in the underlying process (Jennings *et al*., 2001).

In a study of children with a history of OME between two and four years of age who had otological examination at the age of 8, ear examination revealed various otological sequelae; atrophy, atelectasis, tympanosclerosis, and attic retraction (Schilder *et al*., 1995). These abnormalities were more prevalent in children who had had treatment of OME in the form of ventilation tube insertion. This is likely to be the result of the chronic middle ear inflammation that necessitated the treatment as similar changes are seen in children with OME who have never had grommets. If the changes were attributed to the surgery alone, then one would expect only part of the eardrum around the ventilation tube to be affected but they are found to affect the whole of the eardrum implying a generalised middle ear chronic inflammatory process.

1.2 Aetiology of OME

Understanding the patho-physiology of a disease is the first step towards providing an effective treatment. The aetiology of OME is still unclear with many theories proposed, and this is reflected by the multiple treatment strategies that are employed, some of which are based on entirely anecdotal evidence. What is known so far is that the middle ear is an end organ lined with modified respiratory epithelium with the ability to produce mucin in response to several types of local irritants. Mucin overproduction causes a middle ear effusion with consequent hearing loss. This section will examine the evidence for and against the various theories.

1.2.1 Composition of Middle Ear Effusion (MEE)

Although the accumulation of MEE is a by-product of disease, the healthy middle ear constantly secretes fluid that does not result in middle ear effusion. This suggests differences in the composition of the fluid in health and in disease and to the body's different ability to clear fluid in the two states. As the middle ear is in continuity with the nasopharynx via the eustachian tube and is lined with respiratory epithelium, the secretions resemble those of other respiratory mucosal surfaces. Mucosal epithelial cells cover most of the upper respiratory tract, and act as a barrier between the host and the outer environment. Mucus acts as an important mechanical transduction mechanism by trapping bacteria and debris into a matrix containing a rich array of antimicrobial molecules - mucins, antibodies, defensins, protegrins, collectins, cathelicidins, lysozyme, histatins, and nitric oxide (Linden *et al*., 2008). Protegrins are antimicrobial peptides containing 16 to 18 amino acid residues, and are found in abundance in mucus. They act by disrupting bacterial or fungal cell membranes through the formation of pores thus causing cell death. They have widespread activity against gram-positive and gram-negative bacteria and yeasts, and kill bacteria typically within minutes (Ostberg and Kaznessis, 2005). Protegrins and other antimicrobial peptides therefore form a vital part of local host immunity in the middle ear.

In addition, mucus acts as a physical barrier by forming a highly hydrated gel layer, the thickness of which depends on the mucosal site. Mucus covering the eye is thinner than in the stomach. Mucus is not static and is in constant motion with the help of beating cilia. This movement helps clear trapped dust particles in the mucus that travel out of the middle ear via the eustachian tube to the nasopharynx. Nasopharyngeal secretions are ultimately swallowed into the stomach where the highly acidic environment is bactericidal.

In disease, fluid accumulates in the middle ear, and the most widely performed procedure to treat OME is the surgical drainage followed by the insertion of a ventilation tube. The fluid is suctioned and then disposed of. The readily availability of this fluid has meant that a large amount of research has been performed to study its contents in the hope of shedding some light on the pathogenesis of OME. However the ready availability of this by-product for research might not mean that this is the best material to examine, as sometimes less readily available material such as middle ear mucosa provides more clues. Nevertheless the wealth of information gained from examining MEE's cannot be ignored and must be taken into consideration.

MEE's are composed of mainly water. Within the fluid there are cells, cell debris, electrolytes, and high molecular weight compounds. The high molecular weight compounds are composed mainly of mucins, in addition to smaller amounts of proteins, lipids, and DNA. Mucins provide the physical characteristics of middle ear secretions and will be looked at in greater detail in the following section.

1.2.2 The Role of Mucin

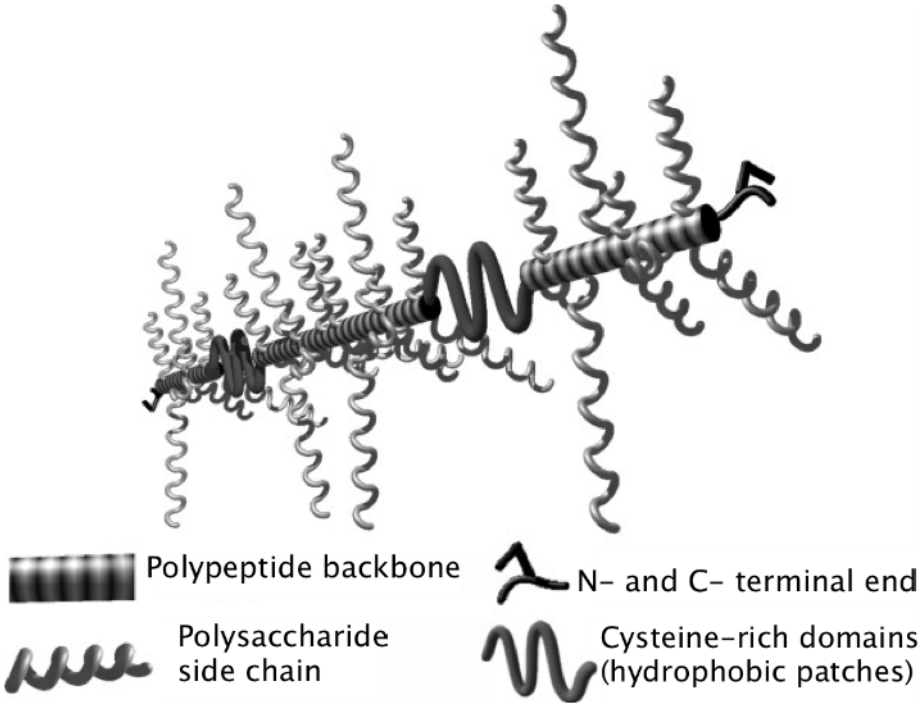


Figure 1.2 Molecular structure of mucin showing a polypeptide protein backbone with polysaccharide side chains giving a bottlebrush appearance. http://www.cardiff.ac.uk/chemy/staffinfo/SMG/mucin.jpg

The main constituents of mucus are mucin glycoproteins. Mucins are high molecular weight compounds composed of a protein backbone with polysaccharide side-chains, and are interconnected with di-sulphide bonds. This molecular structure gives mucins a 'bottle-brush' appearance (Figure 1.2). Mucins are responsible for the viscous properties of mucus helping to lubricate, prevent dehydration, and protect the underlying mucosa. There are many mucin genes expressed in the body, the ones identified and expressed in middle ear epithelium biopsies of children with OME being the following; *MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC7, MUC8, MUC9, MUC11, MUC13, MUC15, MUC16, MUC18, MUC19,* and *MUC20* (Kerschner 2007). These can be subdivided depending on the function of their peptide products as follows: secretory gel forming - MUC2, MUC5AC, MUC5B, MUC19 (Hutton *et al.*, 1997, Ubell *et al.*, 2010), secretory non-gel forming - MUC7, and cell surface mucins - MUC1, MUC3A/B, MUC4, MUC13, MUC15, MUC16, MUC20 (Linden *et al.*, 2008). MUC8 is as yet unclassified though high levels seem

to be associated with increased ciliogenesis of the middle ear effusion and is increased in the ciliated cells of the middle ear (Choi *et al*., 2005). MUC9 is linked to important epithelial functions and MUC11 is linked to cell adhesion and bacterial pathogenesis.

Gel-forming mucins are the main constituents of mucus and are responsible for providing their visco-elastic properties. Increased expression of MUC5AC, a major gel - forming mucin in OME, is found to be associated with increased viscosity of middle ear effusion (Ubell *et al.*, 2010). Cell surface mucins are present on the apical membranes of mucosal epithelial cells. They are thought to provide a barrier to other molecules to attaching to the cell membrane. They are also thought to have intracellular functions, regulating growth and apoptosis when presented with microbes or toxins (Linden *et al.*, 2008).

Mucus provides attachment sites for bacteria to adhere to thus preventing bacteria from invading the underlying mucosa to cause an infection (Reddy *et al.*, 1987). Bacterial adhesins bind to the oligosaccharides on mucin molecules, and whether this favours the host or the bacteria is debatable as this may well be a symbiotic relationship. Furthermore, epithelial mucus secretions contain immunoglobulins, lysozyme, lactoferrin and complement components all of which contribute to the host innate immunity. However if all these defensive mechanisms were to fail then an acute suppurative otitis media would ensue (Kubba *et al.*, 2000).

Given that MUC5AC functions as a primary innate defence mechanism, changes to the structure of the molecule may have a role in predisposition to OME and other middle ear infections. In support of this theory, it has been found that OME patients are more likely to have subtle genetic differences in the *MUC5AC* gene when compared to controls; Sixty percent of patients with OME carried a longer allele named as *MUC5AC-b*, compared to thirty three percent of controls, p=0.055 (Ubell *et al.*, 2010). It is also currently not known whether this allele is associated with persistent OME into adulthood and this could be an area for future research.

1.2.3 Inflammatory aetiology; Link between Mucins and Cytokine activity

One study on MEE found compositional differences between effusions. This was the case between different children with OME in addition to the same child with bilateral OME having different composition between left and right ear (Johnson *et al.*, 1997). The concentrations of mucins and cytokines IL-8, TNF-α, and IL1-β were also studied and it was found that thicker viscous effusions due to higher concentration of mucin also had higher levels of cytokines. This finding gives an important aetiological clue that local inflammatory factors are at play. Animal studies have shown that an inflammatory stimulus such as bacterial endotoxin stimulates the production of TNF-α, and this leads to mucin production and mucin hyperplasia (Hunter *et al*., 1999). More importantly, this effect has been reproduced in vivo with human mucin-producing goblet cells showing an increase in production of cytokine IL-8, MUC5AC, and MUC5B following exposure to bacterial lipo-polysaccharide (LPS) (Smirnova *et al*., 2003).

Vascular proliferation of middle ear mucosa, infiltration of plasma cells and lymphocytes, metaplasia of the middle ear epithelium, proliferation of goblet cells and mucus glands, and the presence of IgA and IgG in higher levels than in serum have been observed in the middle ear effusion (Ishii *et al.*, 1980, Sade 1966, Jones *et al.,* 1979). One important inflammatory mediator that has emerged as an important element is nitric oxide (NO). NO is implicated in many inflammatory - mediated diseases such as asthma, nasal polyposis, Crohns disease, and rheumatoid arthritis. It functions as a signalling molecule, but when released in large quantities it has cytotoxic actions (Anggard, 1994). *In vitro*, NO has been shown to increase mucin production in cell cultures of goblet cells that principally produce MUC5AC, which is one of the important mucins identified in the middle ear (Capper *et al.*, 2003), and it would be interesting to see the effect of NO on the various alleles of *MUC5AC* gene identified in molecular studies. Similar findings have been seen *in vivo* using animal models where NO has been found to increase the production of mucin (Rose *et al.*, 1997). NO metabolites have been found in middle ear effusions suggesting that it is an important mediator in OME (John *et al.*, 2001).

Further evidence of an inflammatory aetiology has arisen from the work of Grote and colleagues on rat middle ears and MEE from humans (Nell *et al*., 1999 a, b, and c). Human MEE classified as mucopurulent contained higher levels of lipopolysaccharide endotoxin and TNF-α. In an *in vivo* study by the same group, endotoxin was found to induce proliferation of human middle ear epithelium, increase the number of secretory cells, and increase the number and length of microvilli, thus supporting other studies previously mentioned. Many research groups have looked at the links between inflammatory factors and OME adding to our knowledge of the underlying aetiology. The following step is to consolidate this wealth of evidence to try and clarify the sequence of events that occur in OME. Kubba *et al*. (2000) have comprehensively reviewed the current position and have postulated steps in pathogenesis that are displayed in a flowchart (Figure 1.3).

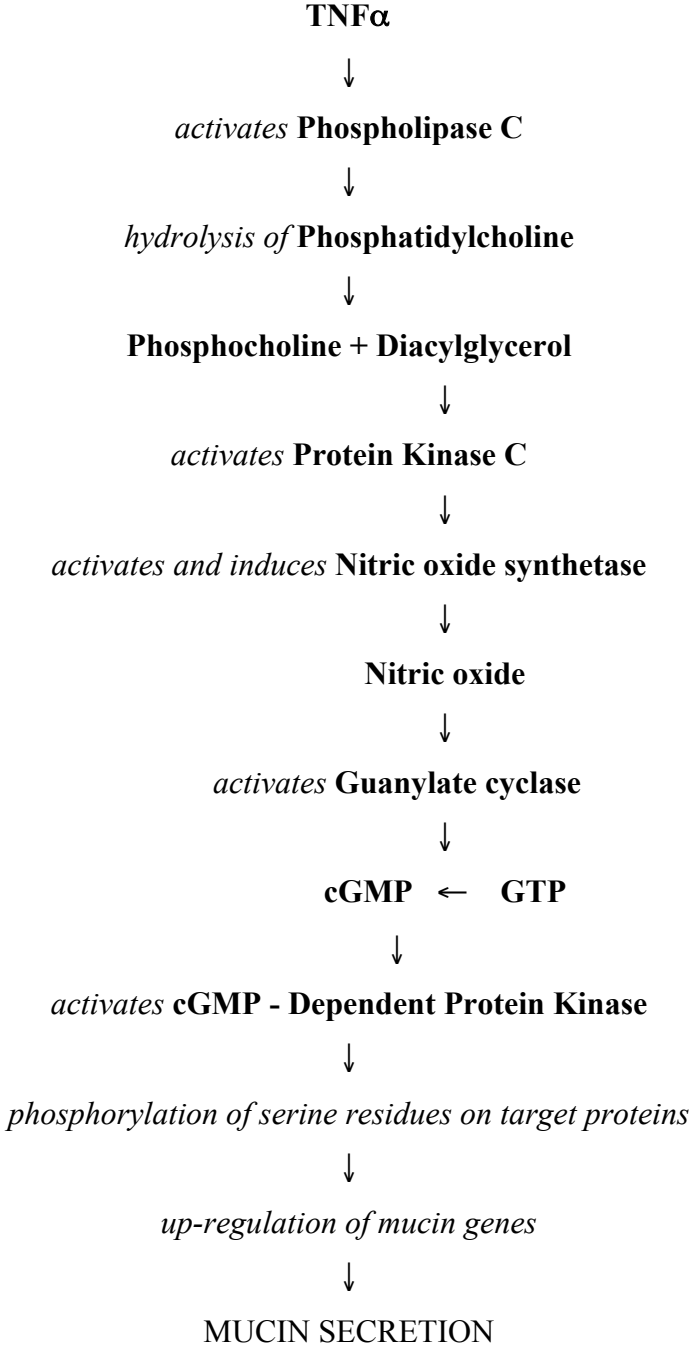


Figure 1.3 A putative pathway for the stimulation of mucin secretion (Kubba *et al*., 2000)

With the overwhelming evidence of an inflammatory aetiology the debate shifts to what is causing this inflammation. Several factors that may cause the production of inflammatory mediators leading to excessive mucin secretion are discussed below.

1.2.4 Viruses

As the immune system of children develops, they are prone to various upper respiratory tract infections including viruses, manifesting clinically most commonly through the symptoms of the common cold. Respiratory syncytial virus, rhinovirus, influenza virus types A and B, and adenovirus are common culprits. One would expect that viruses affecting the upper respiratory tract would also affect the middle ear, and in fact viruses have been identified in middle ear effusions (Pitkaranta *et al.*, 1998). Viral infections can cause inflammation of the middle ear mucosa resulting in increased production of mucin through mechanisms previously described causing a middle ear effusion. This is likely to be the case in patients who have resolution of OME in a short period of time. As viruses are short-lived, it is expected that the removal of the inflammatory source would cause the reversal of the middle ear effusion. It is difficult to prove or disprove the importance of viruses in the aetiology of OME however there has been no convincing evidence in the literature to suggest an important association in cases of chronic OME.

1.2.5 Atopy

Allergy and atopy have been suggested as a potential aetiology though atopy is just as prevalent in normal children as in children with OME (Tos *et al.*, 1979). A systematic review of previous drug trials found that there was no benefit in taking oral anti-histamines for OME (Griffin *et al*., 2006). Given this evidence, currently the general opinion is that atopy has little or no role in the aetiology of OME.

1.2.6 Eustachian tube dysfunction and the ‘ex vacuo’ theory

Anatomically the eustachian tube functions as a passageway for the clearance of mucus from the middle ear. It is lined with respiratory-type epithelium with beating cilia to help the movement of mucus to the nasopharynx. Pathology affecting this process such as in the rare condition of Primary Ciliary Dyskinesia will result in an

increased incidence of OME (Bush *et al*., 2007z). This cannot however be generalised to most children with OME who have normal ciliary function. Historically obstruction of the eustachian tube was thought to cause negative pressure in the middle ear with oxygen being absorbed. The negative pressure was thought to cause a transudate that filled the middle ear. The 'ex vacuo' theory has largely been abandoned for a number of reasons: middle ear effusion display properties of an exudate, and there is metaplasia in the middle ear with evidence of ongoing inflammation. An increased concentration of inflammatory cytokines in the effusion is associated with increased viscosity and chronicity of OME (Johnson *et al*., 1997). This may impair ciliary function and muco-ciliary clearance resulting in functional obstruction.

Eustachian tube dysfunction may cause OME in other ways that are more plausible than the 'ex vacuo' theory. Children with gastro-oesophageal reflux are at a higher risk of developing OME, and in fact pepsin concentrations in middle ear fluid at concentrations up to 1000 times greater than that in serum have been reported (Tasker *et al.*, 2002). Dysfunctional eustachian tubes may not be effective in stopping the reflux of stomach contents, leading to inflammation. Eustachian tubes in children are different from those in adults as they are more horizontal and less developed allowing influx. In order to investigate this theory, in vivo studies may need to be performed to test for the presence of undigested food products in the eustachian tube. Although stomach acid is expected to eradicate most bacteria present in food, it is not unreasonable to expect a decay of left over food products by upper respiratory tract bacteria given this is outside the low pH environment of the stomach.

1.2.7 Bacteria

Although the previous factors discussed may contribute to the development of OME, there is overwhelming evidence that a bacterial infection is the major underlying cause in most cases. ENT surgeons were prompted to consider a bacterial aetiology by the high degree of overlap between acute otitis media (AOM) and OME. Children with OME have five times the number of AOM episodes as other children, and 50% of middle ear effusions follow an episode of AOM (Alho *et al.*, 1995). Most of the risk factors for OME previously identified can be associated with an increased risk of bacterial infection. Day care attendance, large number of siblings, and low socio-economic group may increase the chance of being in a less hygienic environment where the risk of contamination with bacteria from other children or the environment may be higher. Bottle-feeding confers reduced immunity when compared to breast-feeding, and can increase the susceptibility to bacterial infections. Snoring may be due to large adenoids in the nasopharynx. Adenoids are lymphoid tissue that may be implicated in upper respiratory tract infections and thus bacteria on the surface of adenoids may spread through the eustachian tube into the middle ear to cause an otitis media. This is supported by a study showing a significantly higher number of pathogens and species in the nasopharyngeal flora of children with OME than that of other children (Hemlin *et al.*, 1991). This in itself is not solid evidence to prove a direct cause of the OME, but gives weight to this theory. An in vivo study to observe the effects of inoculating the nasopharynx with pathogens may prove a direct cause if there was subsequent development of OME.

More conclusive evidence has arisen from bacterial culture and PCR analysis of fluid from middle ear effusions of children with OME. With conventional culture techniques of middle ear effusions from chronic and recurrent OME, excluding AOM, the highest culture rates reported were approximately 35% (Giebink *et al.*, 1982, Stanievich *et al.*, 1981). Interestingly, PCR analysis of middle ear effusions showed that approximately 80% of effusions contained bacterial DNA (Post *et al.*, 1995). Paluch-Oles et al., (2011) demonstrated similar findings with bacterial growth in 40% of OME effusions using conventional culture technique, but when PCR analysis was performed bacterial DNA was present in 98% of cases. DNA might

simply be the remains of bacteria long after they have died, but mRNA on the other hand has a short half - life and may be a more reliable indication of viable bacteria. Rayner et al (1998) found that 43% of middle ear effusions were positive for *Haemophilus influenzae* mRNA compared to 12% positive on culture, i.e. 35% of culture-negative effusions contained mRNA representing live bacteria. This large discrepancy between culture-positive and PCR-positive effusions has led investigators to suggest that bacteria in the middle ear might be in the form of biofilms, this being due to the fact that biofilms have slow growth rates and therefore are difficult to detect using standard culture techniques.

Given the evidence of bacterial DNA and mRNA in the middle ear, the likely explanation is the presence of live bacteria in the majority of cases of OME. Dead bacterial products may also play a role in the pathogenesis. *Staphylococcus aureus* peptidoglycans (major structural components of the bacterial cell wall) were found to cause inflammation and organ injury in rats (Wang *et al.*, 2004), and in theory may similarly cause persistent local inflammation in the middle ear following resolution of a middle ear infection.

1.3 Treatment strategies

1.3.1 Medical management

Otolaryngologists have searched for a medical treatment for OME to avoid surgery and its complications. The advantages of a medical treatment become more apparent when dealing with a paediatric population where the number of visits to hospital and interventions can have great impact on patient's lives. The numerous trials that have been performed will be reviewed in the following section regarding their efficacy.

1.3.2 Nasal topical steroids

As OME was considered to be secondary to eustachian tube obstruction, administering steroids that would reach the postnasal space was postulated to reduce inflammation and swelling around the eustachian tube opening, thus relieving blockage. Indeed many clinicians to this day give topical intranasal steroids for patients with OME based on anecdotal evidence. A study in 1982 found no difference between aerosolized nasal steroids and placebo (Shapiro *et al.*, 1982). More recently a double-blind randomised control trial was carried out to test the efficacy of nasal steroids, and 217 children between the ages of 4 and 11 were randomised to either mometasone furoate or placebo for 3 months. There was no clinically significant difference in resolution of OME (Williamson *et al.*, 2009). To confirm these findings a comprehensive Cochrane review of the literature came to a similar conclusion (Simpson *et al.*, 2011). NICE guidelines have since advised against the use of topical nasal steroids in OME, as there is no proven benefit (NICE 2008).

1.3.3 Systemic steroids

As OME is an inflammatory condition of the middle ear mucosa leading to mucus secretion it might make sense to treat with systemic steroids, but studies have had conflicting results. Bluestone and colleagues have led many of the early studies on the medical treatments used in OME. In a recent comprehensive meta-analysis by this team, the effect of systemic steroids and oral antibiotics versus oral antibiotics alone was examined. OME resolved sooner with combination therapy compared with antibiotics alone at 14 days of treatment, but two weeks after finishing treatment there was no difference between the two groups (Mandel *et al.*, 2002). A systematic review of the literature has also shown that while oral steroids, especially when used in combination with an oral antibiotic, lead to a quicker resolution of OME in the short term, there is no evidence of longer-term benefit and no evidence that they relieve symptoms of hearing loss (Simpson *et al.*, 2011). The short-lived effect of oral steroids and antibiotics limits their usefulness clinically, though it does point to an important aetiological clue: OME is not just an inflammatory condition on which steroids would be expected to have an effect, but an infective condition with an inflammatory response hence the quicker resolution with combination of antibiotics and steroids.

1.3.4 Oral antibiotics

Similar to topical and systemic nasal steroids, with any benefit confined to the first two weeks, after which therapy does not seem to make a difference, systematic reviews of the literature have found similar results for antibiotics alone. One of the first meta-analyses of multiple studies between the periods 1966-1991 showed that there was no difference between antibiotics and placebo if patients were followed up for long enough (Rosenfeld & Post, 1992). A more recent meta-analysis incorporating newer studies has reached similar conclusions (Williamson, 2011), and therefore NICE guidelines have not advocated the use of oral antibiotics in OME (NICE 2008).

1.3.5 Other medical treatments

Though both otolaryngologists and parents of children with OME would prefer to avoid surgery for the treatment of OME, no medical treatment has been shown to be effective. Many other conservative approaches such as nasal decongestants, oral mucolytics, and even homeopathy have been tried, but none of these has been shown to be effective (Griffin *et al.* 2006, Cantekin *et al.*, 1983, Commins *et al.*, 2000, Harrison *et al.*, 1999). One particular therapy, autoinflation, based on increasing pressure in the post-nasal space to open the eustachian tubes, has been shown to improve the likelihood of resolution from OME. This adds weight to the eustachian tube dysfunction theory where strategies aimed at improving mucociliary clearance of the middle ear into the nasopharynx may reduce bacterial load and hence resolution of OME. However, compliance is an issue in young children and this limits its use considering that most children with OME are young (Williamson, 2011).

Hearing aids are considered by many to be an acceptable management of OME to improve children's hearing until OME resolves. Hearing aids however are not without their problems; there is potential for noise trauma when OME resolves, along with the risk of battery ingestion, non-compliance with wearing them, and a higher incidence of bullying at school (Leff, 1999).

1.3.6 Surgical Management

As previously mentioned under prognosis, OME in half the cases will have resolved by 3 months. It is therefore currently common practice for children to have a watchful waiting period with documented conductive hearing loss of 25 decibels or worse in the better hearing ear over a 3-month period prior to offering surgery. This has been the formal advice by both NICE and the ENT UK professional body that represent Otolaryngologists in the UK (ENT UK 2009, NICE 2008). In patients with persistent OME after the watchful waiting period, surgery is the most effective treatment providing instant improvement in hearing. Surgery for OME in the form of grommet insertion is therefore the most widely performed procedure in paediatric

otolaryngology; in the year from 2009 to 2010 there were 33,920 operations to drain middle ear fluid in children below 14 years of age in England alone (hospital episode statistics-HES online database).

1.3.7 Grommet surgery

Grommet surgery is performed by initially draining the middle ear fluid via an incision (myringotomy) made through the tympanic membrane, aspirating the fluid, and then inserting a ventilation tube (grommet), which keeps the middle ear ventilated, and equalises pressure between the outer ear and middle ear (NICE 2008). The ventilation tube typically lasts from 9 to 12 months after which it self-extrudes. Some argue about the need to place a ventilation tube if the fluid has been aspirated, but a systematic review of three trials showed that myringotomy and aspiration alone is not effective in improving hearing to an adequate level (Freemantle *et al*., 1992).

There have been over thirteen trials examining the efficacy of grommet surgery. Many of those trials randomise ears of patients rather than the patients themselves. This presents problems in analysing the data, as patients should be taken as a whole. In addition to this, there are varying non-stringent audiometric criteria for including patients into trials. To correct this, many renowned otolaryngological departments carried out a large multi-centre trial (Trial of Alternative Regimens in Glue Ear Treatment – TARGET) using strict audiometric criteria and randomising patients rather than ears (MRC: TARGET 2003). The results of previous trials in addition to the MRC trial have been consolidated in a comprehensive Cochrane meta-analysis (Browning *et al*., 2010) which found that grommets offer on average 10 to 14 decibel improvement in hearing at 3 months after insertion. Hearing benefits continued for 6 months by which time usually the OME resolved when the grommets self - extruded.

Although ventilation tube insertion is currently the gold standard treatment for OME, the mechanism of action is not well understood. This may reflect the failure to understand the aetiology of OME. Part of the explanation may be due to laws of physics with a similar analogy being the inverse water-filled glass and paper

experiment. Glue would not travel down the eustachian tube if the atmospheric pressure acting against the lower end of the glue in the nasopharynx were greater than the pressure of the pocket of air trapped in the middle ear. Myringotomy in theory would equalise the pressure between the middle ear and nasopharynx allowing for the free drainage of glue. Additionally, there are other forces to consider; surface tension and viscosity are two thermo-physical properties that have a linear relationship using the equation proposed by Pelofsky:

$$\ln\sigma = \ln A + \frac{B}{\eta}$$

The symbol ln represents the natural log, A and B are constants, σ is the surface tension, and η is the viscosity. The relevance is that the more viscous the glue is in chronic OME, the higher the surface tension giving the surface of glue an elastic strength that resists disruption. Viscosity on the other hand provides liquids resistance against movement or deformation, this is again relevant when considering that a viscous glue would be harder to clear from the middle ear through the eustachian tube due to the resistance against movement provided by the increased viscosity. A further biological explanation may be due to the drying effect of ventilating the middle ear. If the bacterial aetiology is considered, then bacteria generally prefer warm and moist conditions. Ventilating the middle ear may cause a cooling and drying effect that is less favourable for bacterial replication thus providing an advantage for the local innate immune mechanisms in the middle ear to eradicate bacteria. This is unlikely to be from direct bacterial killing such as that seen in industrial desiccation to sterilise products, the middle ear will always retain a degree of warmth and moistness given it is an internal body cavity. This theory is not free from flaws, as bacteria are known to develop resistance patterns when exposed to unfavourable conditions. Nevertheless, each of the physical and biological theories proposed by the author of this thesis may have a role in the resolution of OME from ventilation tube insertion. In order to prove or disprove these theories in vivo experimental studies need to be performed to provide a better understanding on resolution of OME.

1.3.8 Adenoidectomy

Adenoids are lymphoid tissue located in the postnasal space between the two eustachian tube openings. It was previously thought that removing the adenoids would allow better ventilation and relief of obstruction to the eustachian tube openings thus helping to resolve OME. Nowadays it is thought that the benefit derived from removing the adenoids is because they harbour bacteria that would predispose to infection. Conventionally, adenoidectomy alone was the surgical treatment of choice in OME until the introduction of ventilation tubes (Browning, 2008). On its own, adenoidectomy does not confer as much benefit when compared to grommet insertion in terms of hearing improvement, and therefore it is mainly performed as an adjunct to grommet insertion. Data from the TARGET trial showed there was an additional benefit of 2.3 decibels hearing at 1-year follow-up when compared to grommets alone. The main benefit however is that patients are less likely to have recurrence of OME if an adenoidectomy has been performed concurrently; the proportion of children requiring re-insertion of grommets for recurrence was reduced from 47 to 28 percent (Browning, 2008). It is currently a clinical decision as to which patients should receive concurrent adenoidectomy, and it is common practice that patients developing recurrent OME are advised to have adenoidectomy.

1.3.9 Failure of current treatment strategies

Many of the medical treatments previously discussed have failed to treat OME. Even with the gold standard treatment of grommet insertion, a quarter of patients will develop recurrence of OME necessitating revision surgery after the grommets extrude (Gates *et al.*, 1987). This points to important aetiological clues. As discussed under bacterial aetiology, there is growing consensus that OME is the result of a bacterial biofilm infection. It is therefore not surprising that there is such a high rate of recurrence after grommet insertion; Surgery involves suctioning most of the mucin-containing effusion on which biofilm colonies exist but this inevitably leaves some mucin behind with the potential for biofilm re-proliferation. In addition, biofilms established on the surface of middle ear mucosa are not dealt with. When

one understands the nature of biofilms, it is possible to appreciate why antibiotics for OME have limited short-term efficacy with doubtful long-term results. In order to develop an effective treatment for OME it would make sense to learn about biofilms and make use of the vast knowledge and expertise gained from dealing with biofilms in other areas such as industry and medical device - related infections.

1.4 Biofilms

1.4.1 Evidence of a biofilm infection in OME

The strongest evidence so far linking bacterial biofilms and OME comes from a study by Hall-Stoodley et al. (2006) where middle ear mucosa biopsy specimens were taken from children undergoing ventilation tube insertion for OME. When the mucosal specimens were examined under confocal laser scanning microscopy, 92% of cases showed live bacterial biofilms using live - dead stain. Control specimens from patients undergoing cochlear implants did not show evidence of bacterial biofilms. In addition, all effusion samples obtained were PCR - positive for a bacterial pathogen. Similarly, Paluch-Oles et al. (2011) examined effusion samples following ventilation tube insertions in children with OME. Bacterial growth was demonstrated in 40% of patients with conventional culture technique, but when PCR analysis was performed bacterial DNA was present in 98% of cases. These results agree with work performed in the Nottingham - based Biomaterials - Related Infection Group (BRIG), using CLSM and extended culture techniques to demonstrate live bacteria in 92% of effusions (Daniel *et al*., 2012 - b).

Interestingly, adenoids from children with OME have been shown to have a higher percentage of the mucosal surface covered with biofilm, 27.7%, than those of children with obstructive sleep apnoea (OSA), 0.10%, considered as controls (Hoa *et al.*, 2010). Patients with recurrent acute otitis media (RAOM) however had the highest percentage of biofilm covering the adenoid surface, 97.6%, compared to controls, 0.10% ($p<0.0001$). The methodology was based on scanning electron microscopy (SEM) of the adenoid mucosal surface and computer software to calculate the thickness and surface coverage. It is arguable that biofilms need to be cultured on media and colony forming units calculated to get a true representation of biofilm quantity and indeed as to the true presence or absence of biofilm. The results suggest normal flora is free from bacterial biofilm however it is known that mucosal surfaces in the body are lined with symbiotically friendly bacterial flora and bacteria generally exist in a biofilm state. It would have been useful to culture the flora of the

adenoids comparing the controls with the RAOM group to see if there are any differences in the bacterial species. This may have provided clues as to whether there may be a change in the make up of the bacterial population with perhaps pathogenic bacteria being in larger numbers than less pathogenic species. It is possible that during episodes when the host immunity is low pathogenic bacterial colonisation may migrate into the upper respiratory tract and into the middle ear via the eustachian tube forming biofilms thus predisposing to OME and RAOM. These theories cannot be confirmed and further research in this field is needed to draw firm conclusions.

1.4.2 Biofilms in nature and disease

Early observations of bacteria in natural aquatic ecosystems showed that more than 99.9% of bacteria grow in bacterial communities, biofilms, enclosed together in a matrix, adherent to a surface, and are nutrient sufficient. Very rarely do bacteria exist in the free-floating planktonic form. Biofilms cause major problems in water pipes, and it is for this reason that the water industry was one of the first to develop sophisticated methods for sampling, examining and controlling the growth of biofilms (Donlan and Costerton, 2002). It was around the last three decades that the medical and dental specialties increasingly realised the involvement of biofilms in clinical infections (Donlan and Costerton, 2002).

Today it is well recognised that many clinical infections such as periodontitis, chronic prostatitis and those in cystic fibrosis are the result of biofilm infections. In addition to OME, biofilms are increasingly being found in many ear, nose and throat diseases such as chronic rhinosinusitis (CRS), recurrent tonsillitis, chronic suppurative otitis media (CSOM), and middle ear cholesteatoma infections (Vlastarakos *et al*., 2007). In fact, most indwelling medical devices such as catheters, tracheostomy tubes, and long-term ventilation tubes (grommets) are found to harbour biofilms. In the case of prosthetic heart valves, biofilm infections can result in serious life threatening endocarditis. Biofilms are a particular problem in neurosurgical patients with cerebrospinal fluid (CSF) shunts and external ventricular

drainage devices (EVD), and infection is associated with a particularly poor outcome (Bayston *et al*., 2007).

1.4.3 Adherence

The first and most important step of biofilm formation is bacterial adherence to a surface such as cell, mucin, or implanted device. This adherence is affected by properties of the substrate surface and by bacteria - specific characteristics. Donlan (2002) showed that a solid-liquid interface is also a very suitable medium for microbial attachment and growth, and that some rough surfaces enhance microbial colonisation, depending on the nature of the roughness. This is thought to be secondary to decreased shear force and increased surface area. Hydrophobicity of the surface also seems to be an important factor in attachment of bacteria; it is thought to help overcome the repulsive forces between the bacteria and the surface. A conditioning film forms on most inert surfaces when embedded in an organic medium. This is usually made up of polymers that bacteria cell wall proteins can attach to, thus enhancing adherence (Donlan, 2002). It is important to note that the properties of the surface after conditioning are very different from the original inert surface, and this should be taken into consideration when studying bacterial adherence.

Bacteria that are able to attach to surfaces are generally stronger biofilm producers, and this is dependent on cell surface structures that mediate adhesion. Bacteria find their way to the target attachment site by Brownian motion, and once in the vicinity of the substratum there exists repulsive electrostatic energy. It is at this point that bacteria use nanofibres such as pili or flagella, or produce polysaccharides that can bridge the gap between the bacterial cell wall and the substratum due to their small radii that can pierce the energy barrier, thus acting as adhesins (Hori & Matsumoto, 2010).

1.4.4 Polysaccharides

Given the importance of polysaccharides in the initial adherence of bacteria to surfaces and their role as primary matrix components of biofilm a considerable effort has gone into studying the various types. Lipopolysaccharide (LPS), is found only in Gram-negative bacteria and is composed of lipid A, core polysaccharides, and an outermost region of O antigen units thought to be important for the initial bacterial surface adhesion. Extra-cellular polymeric substance (EPS) is the other main group, also named by some, exopolymeric substance. Gram-positive bacteria produce a variety of EPS including teichoic and lipoteichoic acids, capsules and sometimes Polysaccharide Intercellular Adhesin (PIA). EPS can be classified into three types, capsular EPS that is tightly bound to the cell wall, loosely bound EPS that is responsible for the slime of biofilms, and soluble EPS. PIA is the most widely studied EPS and is produced by *Staphylococcus epidermidis* and *S aureus*. The unit of PIA is N-acetyl-glucosamine, which is a monosaccharide derivative of glucose and an amide between glucosamine and acetic acid, essentially a peptidoglycan. Repeat units of N-acetyl-glucosamine (Figure 1.4) are linked in a β-1,6 formation hence its chemical name β-1,6-linked N-acetyl-glucosamine (Figure 1.5). PIA residues typically contain up to 15% de-N-acetylated amino groups (Mack *et al*., 2004).

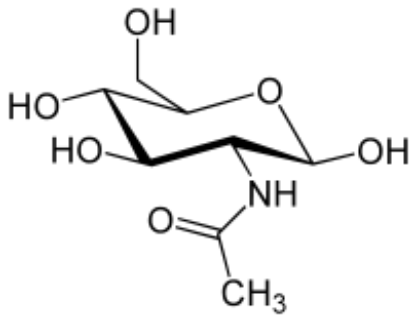


Figure 1.4 Molecular structure of N-Acetyl-glucosamine, a monosaccharide derivative of glucose, repeat units form the backbone of a PIA molecule.
http://upload.wikimedia.org/wikipedia/commons/9/96/N-Acetylglucosamine.svg

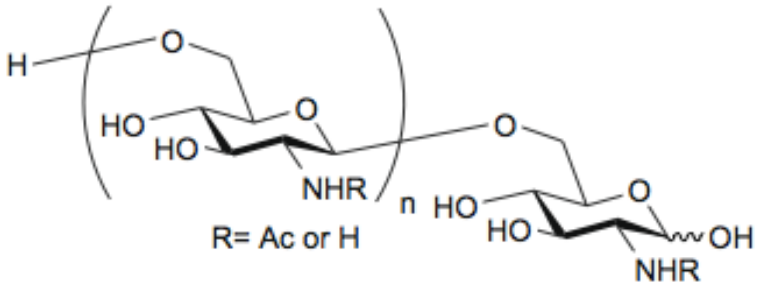


Figure 1.5 Molecular structure of PIA showing the β-1,6 formation of the N-Acetyl-glucosamine unit (Leung *et al*., 2009)

1.4.5 Biofilm interaction with mucin

Biofilm polysaccharides share similarities with the molecular structure of mucins secreted in the body, as both are glycosaminoglycans (GAG's) or peptidoglycans. This similarity in structure gives biofilms their typical slimy mucinous appearance as heavy glycosylation allows for the attraction of water molecules to form gel-like macromolecules. Mucin can act as a substrate for the growth of bacterial biofilms providing a large surface area for bacterial attachment. This is based on the principle that coated surfaces enhance bacterial attachment and is seen similarly in protein-coated surfaces. *Pseudomonas aeruginosa* biofilm attachment to uncoated glass, and glass coated with each of actin, DNA and human mucin has been compared, showing that mucin-coated surface resulted in the growth of highly structured, heterogeneous biofilms with large cell aggregates and increased resistance to antibiotics (Landry *et al*., 2006). It is interesting to note that biofilm grown on actin and DNA- derivatised surfaces was comparable to that of biofilm on glass given that both actin and mucin are proteins. *Ps aeruginosa* is known to have mucin-adhesins that mediate bacterium-mucin interactions such as the flagellar cap protein, and FliD (Carnoy *et al*., 1994, Arora *et al*., 1998). Supporting this, FliD deficient mutant of *Ps aeruginosa* was found to produce similar flat biofilms on mucin surface comparable to that on glass surface (Landry *et al*., 2006). Much of the research in the literature on this topic has been focused on cystic fibrosis patients, but these interactions may also prove to be relevant for biofilm-mucin interactions in the middle ear.

Understanding the similarities of biofilm structure to mucins opens the potential to new avenues for treatment. Chemicals that are known to break down the structure of mucins may be used in similar ways to break down biofilm structures. Ambroxol, a mucolytic, has been shown to enhance degradation of alginate (an exopolysaccharide) produced by *Ps aeruginosa* biofilms resulting in the breakdown of mature biofilms. In addition there was a reduction in the alginate synthesis gene expression (Li *et al*., 2008).

1.4.6 Antibiotic recalcitrance and small colony variants (SCV's)

Bacteria in a biofilm state show marked reduction in susceptibility to antibiotics, and this is thought to be multi-factorial. A theory that has now been largely abandoned is that the gel-like slime layer forms a physical barrier to reduce the penetration of antibiotics to bacteria (Souli & Giamarellou, 1998). Antibiotics are mostly formulated to be water-soluble and the extracellular matrix is 98% water, so antibiotics should be able to penetrate the biofilm matrix thereby nullifying the 'barrier' theory. Rifampicin has been shown to penetrate *S epidermidis* biofilms but fails to kill biofilm bacteria (Zheng and Stewart, 2002).

Antibiotic recalcitrance is now thought to be largely due to the ability of a biofilm to produce bacterial subpopulations in a dormant state known as small colony variants (SCV's) with distinctive phenotypic and pathogenic traits. Characteristically, SCV colonies grown on blood agar are one-tenth the size of their wild-type counterparts, are non-haemolytic, non-pigmented, and show marked reduction in growth rate. SCV's are not unique to biofilms and were described in 1910 with aberrant colonies of *Salmonella typhi* (Proctor *et al*., 2006). A wide range of SCV bacteria have been demonstrated in persistent and recurrent infections such as in cystic fibrosis, osteomyelitis and device-related infections. *S aureus* SCV's are the most extensively studied and serve as a good example to explain SCV mechanisms of other bacteria.

For SCV's to develop, bacteria need to be starved of nutrients triggering defects that affect the ATP-producing electron transport system (Bayston 2000). These conditions are present in the deeper - most layer of a biofilm due to the oxygen and

nutrient gradient but SCV's can exist independently of biofilms given similar conditions. Auxotrophism to haemin and/or menadione are common in SCV's, both being important compounds for the electron transport system. Pinpointing to the exact defects in SCVs and how they bring about the observed phenotype has proven difficult but hypothetical explanations have been provided; ATP is important for cell wall synthesis, and the reduction in amount of ATP is thought to affect growth rate and produce small colonies. Defects in the electron transport system are thought to be responsible for a reduction in the membrane potential, thus affecting the uptake of cationic compounds. This is thought to directly affect the transport of aminoglycosides into the bacterial cell and accounts for their reduced antibiotic susceptibility (Sandberg *et al*., 2011). Carotenoid biosynthesis is directly related to electron transport and reduced synthesis produces non-pigmented colonies (Proctor *et al*., 2006). Haemolysis around colonies grown on blood agar plates is secondary to the lysis of red blood cells from toxins produced by bacteria such as hydrogen peroxide or stroptolysin. SCV colony non-haemolytic appearance is thought to be due to a reduced biosynthesis and uptake of amino – acid and consequently a reduction in non-essential toxins (Proctor *et al*., 1998).

Interestingly these phenotypic characteristics are reversible in a haemin or thiamine (pre-cursor to menadione)- abundant environment. This ability to revert to the parent phenotype is explained by the bypassing of the deficient intra-cellular mechanisms by providing the required nutrient directly from the extra-cellular environment. The underlying genetic defect of *S aureus* auxotrophic SCV's has not been found yet, but genetic studies introducing defects into the electron transport system such as *Hem B* mutant resulted in *S aureus* with similar characteristics to the auxotrophic clinical SCV. These characteristics were reversible on exposure to haemin at a concentration of 1μg / mL (Proctor *et al*., 2006).

In a metabolically active bacterium the cell wall is under constant remodelling with build up of cross-links of peptidoglycans with the help of penicillin-binding-proteins (PBP) and breakdown enzymes that hydrolyse the cross linking. β-lactam antibiotics work by irreversible binding to PBP thus tipping the balance towards breakdown of the cell wall by the continually active hydrolyses of cross links. SCVs have a

reduced rate of metabolic activity and the rate at which the cell wall is remodelled is greatly reduced. This is thought to have a protective effect against β-lactams that depends on a high rate of cell wall remodelling and has been an observed phenomenon from the 1940's (Schnitzer *et al*., 1943). SCV's also have the ability to persist intra-cellularly after phagocytosis thus evading many of the immune mechanisms of the body (von Eiff 2008). Further recalcitrance to antibiotics is observed when SCV's of *S aureus* attach to a fibronectin-coated surface when compared to SCV's in fluid phase (Chaurd *et al*., 1997). This is often the first step prior to biofilm formation. Many of the characteristics of SCV's described resemble those of biofilms, adding further evidence to their importance in biofilm pathogenesis.

1.4.7 Biofilm examination and measurement in research

Direct imaging is the gold standard method for examining biofilms. They reveal a wealth of knowledge about structure and have been used to provide much of the information about biofilms. Scanning electron microscopy (SEM) involves chemical fixation to preserve and stabilise structure, followed by dehydrating the sample and then coating with a conductive material such as gold. SEM requires images to be sampled under vacuum so that electrons directed towards the sample are not affected. Biofilms are very hydrated structures and processes that dehydrate them will cause significant destruction, in addition, the image is an artefact of the preparation process and not the direct image of the object to be examined. This needs to be taken into consideration as poor preparation techniques may result in poor quality images. Despite these disadvantages, SEM gives high-resolution images at very high magnification and has an important role in biofilm research.

Environmental SEM (ESEM) is different from conventional SEM in that it has a gas medium instead of vacuum with the advantage that specimens do not require potentially destructive preparation techniques. This is important when imaging live biological material. The main disadvantage of ESEM is the limitation of the distance between the electron beam emission and the sample, typically a millimetre to 10 millimetres, as larger distances result in most of the electrons scattering through the

gas medium with few electrons reaching the specimen. The arrival of CLSM has seen a revolution in imaging with 3D processing possible and samples can be viewed without any processing, similar to ESEM. CLSM can be used to monitor living biofilms dynamically, although at a lower magnification (120x - 14,400x), and less resolution typically up to 10 micrometers compared to conventional SEM. Although conventional SEM gives better resolution, the image quality generally deteriorates at magnifications above 30,000x.

There are other acceptable and less time consuming methods of examining and quantifying biofilms in research, the most commonly used being based on the plate viable count procedure. This involves removing the biofilm from the surface mechanically by vortexing or sonication. The biofilm bacteria are rendered free-floating and can be cultured on suitable medium; colonies can be counted to quantify the bacteria in the biofilm. This method is useful when measuring the effect of anti-biofilm agents on biofilm quantity. There are many assays described in the literature based on the same principle of plate viable count procedure and each has its advantages and disadvantages.

Another widely used method is the crystal violet assay described initially by Christenson *et al*., 1986 which depends on growing biofilms in a microtitre plate, after which they are stained with crystal violet and the light transmittance is measured spectro-photometrically. This is a fast and reproducible technique, but there have been major criticisms in methodology; firstly, light transmittance through dry, cracked biofilm material at the base of the well cannot be reproducibly measured; secondly, light is directed through the base of the well and any biofilm on the side walls of the wells is not measured; thirdly, it is notoriously non-reproducible; and fourthly, it is susceptible to effects of precipitation through gravity. Since then there has been an important modification (Stepanovic *et al*., 2000) wherein following staining with crystal violet, the dried dye-bound adherent cells at the bottom in addition to the side walls of the wells are resolubilised with acetic acid and measured spectro-photometrically.

1.4.8 Clinical diagnosis of biofilm infections

As biofilms are attached to surfaces in an irreversible fashion, clinically it is very difficult to diagnose biofilm related infections. Although SEM and CLSM are used in research, they are neither cheap nor practical in the clinical setting. One of the cheaper ways of diagnosing biofilms in the laboratory setting is using the plate viable count procedure. This is possible only if the attached surface is available such as the case when an infected indwelling catheter is removed. If the substrate surface is part of the human body then this is not possible such as the case with middle ear mucosa in OME. Bacteraemia or septicaemia is not commonly found in most biofilm infections and blood culture does not help.

Due to the reasons outlined, currently biofilm-related infections are difficult to diagnose and there needs to be an increased drive to find new ways for diagnosis. But with the increasing acceptance of their existence in many clinical infections, it would make sense to treat patients with chronic infections as having an underlying biofilm infection. Given our knowledge of biofilm behaviour, short courses of oral antibiotics are unlikely to be effective. Currently most clinical laboratories calculate antibiotic dosage and susceptibility using minimum inhibitory concentrations (MIC), i.e. minimum concentration of antibiotic needed to inhibit bacteria in planktonic free-floating form. In order to get a better realisation of bacterial susceptibility in chronic infections, biofilm (as opposed to planktonic) inhibition/eradication assays should be used. Bacteria in biofilm typically require 100 to 1000 x MIC of antibiotic concentration compared to planktonic bacteria thus explaining the failure of short courses of antibiotics in treating chronic infections when used at the currently acceptable therapeutic doses.

1.4.9 Therapeutic possibilities in OME

After dissecting the mechanisms of biofilm formation, it is possible to propose potential targets for therapy for OME. High concentration of antibiotics over a prolonged period of time at the site of the biofilms would result in eradication. This cannot be achieved systemically because antibiotic concentrations would reach toxic levels in the body long before achieving adequate levels at the site of the biofilm. As a general principle therefore it would be beneficial deliver antibiotics directly to the site of the biofilm thus avoiding systemic toxicity. The middle ear is anatomically difficult to access and an intact tympanic membrane is a barrier to drug delivery into the middle ear. It would therefore be beneficial to invest research into novel methods of drug delivery to the middle ear. Currently there is ongoing research in magnetically directing nanoparticles that are ionically charged through an intact tympanic membrane (Shapiro *et al*., 2010), however these are still at an experimental stage and not widely available.

Another potential therapeutic possibility is the disruption of biofilm structure through the use of mucolytics as discussed earlier. This exploits the similarities in the GAG structure of biofilms to mucins. As the first step of biofilm formation is adherence to the substrate surface, many research groups have attempted to alter the surface characteristics to reduce the likelihood of adherence but this is possible only when designing indwelling medical devices, because tissue surfaces such as the lungs in cystic fibrosis are not alterable.

1.5 Proposed Solution

1.5.1 Delivery of anti-biofilm agents to the middle ear

Local drug delivery to the middle ear affords advantages over systemic treatment: the drug is delivered only to the target site reducing the amount of drug needed, higher concentrations can be achieved locally, systemic side–effects are avoided and bacterial resistance risk is reduced. This is feasible at ventilation tube insertion and makes surgery only marginally more invasive; the surgeon would need to initially make a myringotomy incision on the tympanic membrane, and the drug can then be delivered through the incision after which the ventilation tube can be inserted. The use of a slow-release system would ensure longer-term delivery of the antibiotic, and if the system were biodegradable, it would not have to be removed when depleted. A slow-release preparation would therefore ensure that the prescribed dose was given over an optimum time interval.

1.5.2 Treatment strategy under development

We have developed a biodegradable copolymer scaffold consisting of poly-lactic-co-glycolic acid (PLGA) into which antibiotics are introduced giving controlled release over a period of time. Over 3 weeks this scaffold breaks down to the normal metabolites, lactic acid and glycolic acid, which are absorbed and metabolised by the body. The scaffold has been designed with a cylindrical shape and is hollow in the middle, to decrease the chance of eustachian tube blockage if it were to travel down the eustachian tube and stay there until it is broken down. The scaffold would incorporate the anti-biofilm drugs.

1.5.3 Antibiotics

The antibiotics rifampicin (Rif) and clindamycin (Clind) have been chosen from knowledge of their efficacy against the bacteria usually present in the middle ear biofilms (Daniel *et al*., 2012 - b), and for their low ototoxicity profile in view of the prolonged nature of antibiotic release. Bacteria usually develop resistance to rifampicin when used on its own, but we have demonstrated that when combined with another carefully chosen antibiotic such as clindamycin, mutational resistance does not arise (Bayston *et al*., 1989).

1.5.4 Mucolytics

Published data suggest that some mucolytics have anti-biofilm action and therefore may be used as an adjunct in the scaffold. Disruption of the biofilm could be expected to reduce the requirement for high dose antibiotic and to increase the chance of eradication of the biofilm bacteria.

1.5.5 N-Acetylcysteine as an adjunct to antibiotics for the eradication of biofilms

N-Acetylcysteine (NAC) is a mucolytic with established anti-biofilm properties and an excellent safety profile (Aslam *et al*., 2007). It has been shown to have a synergistic effect when used with antibiotics in vitro where biofilms are implicated, involving catheters and stents (Aslam *et al*., 2007, El-Feky *et al*., 2009, Venkatesh 2009), as well as in vivo for the eradication of *Helicobacter pylori* (Cammarota *et al*., 2010). There is a scarcity of studies looking at the mechanism of action of NAC on biofilms. A search of the literature identified one study that looked at various effects of NAC on biofilms. NAC was found to inhibit bacterial attachment to stainless steel and caused detachment of attached bacteria. NAC was shown to reduce the production of extracellular polysaccharide (EPS) in starved bacteria but there was no direct degradation of EPS extracted from various bacteria by NAC

(Olofsson *et al*., 2003). We postulate that the biofilm reducing potential of NAC renders bacteria into the planktonic state thus allowing antibiotics to eradicate bacteria more easily and this may be responsible for the observed synergy in the studies mentioned.

NAC is of interest in OME research as it also disrupts mucins. Mucus is the substrate on which biofilms form, and is the major component that causes viscosity of the middle ear effusion. NAC breaks the S-S bonds thus reducing the polymeric mucin to a much less viscous monomeric compound. Disruption of mucin may remove the surface to which biofilms attach thus destabilising the biofilm and allowing better antibiotic efficacy. The direct application of NAC into middle ears of children at time of ventilation tube insertion has been shown to reduce recurrence rate of OME (Ovesen *et al*., 2000). It has to be noted however that despite the NAC intervention the recurrence rate was 13.5%; although the biofilm matrix is disrupted by NAC, there is no anti-bacterial killing effect without the presence of antibiotics and biofilm colonies are likely to re-form causing recurrent OME.

1.5.6 Broad project aims

NAC is emerging as a promising mucolytic agent with biofilm reducing properties. One aim of this project is to explore the potential use of NAC as an adjunct to antibiotics in the middle ear in the context of OME, as recent work from our group has shown (in vitro) that antibiotics (clindamycin and rifampicin) on their own need to be present in extremely high concentrations (1000 x MIC) for a prolonged period of time in order to eradicate biofilms (Daniel *et al*., 2012 - a). The aim is to reduce the concentration of antibiotics needed to eradicate biofilms by using NAC concurrently. The effects of NAC on biofilm extra-cellular matrix will be examined with particular regard to the mechanisms of action. In order to use NAC at safe levels in the middle ear, optimal concentrations that result in synergy with antibiotics will be investigated. The results of this study may be used in future for the ongoing design of a biodegradable drug delivery pellet to deliver biofilm-reducing agents to the middle ear.

Section 2. Project Outline

Bacterial identification

Test bacteria for use in the experiments were identified from bacteria isolated from middle ear effusion (MEE) of OME children who have undergone myringotomy and aspiration (Daniel *et al*., 2012 – b). Following this the optimal environments for these bacteria were tested.

Biofilm production

To select suitable bacteria, the modified microtitre test plate - crystal violet assay (Stepanovic *et al*., 2000) was used to classify bacteria as weak, moderate, or strong biofilm producers. The strong biofilm producers were used for the biofilm experiments.

Proof of concept

To see if NAC enhances biofilm eradication when combined with antibiotics, a silicone disc biofilm assay was used. This assay was based on the plate viable count method and has been developed and validated by the Biomaterials - Related Infection Group (BRIG) previously (Daniel et al., 2012 – a).

Method of action of NAC

To investigate the mechanism of action of NAC, extra-cellular slime was extracted and isolated from bacteria using the extra-cellular slime assay previously developed and verified by Bayston and Rogers (1990). Once isolated, extra-cellular slime was then exposed to NAC under various conditions. To determine the effect of exposure to NAC, the quantity of extra-cellular slime was measured with and without NAC exposure using a modification of the alcian blue quantification assay (Whiteman 1973, Burlingame et al., 1981). To further determine changes in the size of the extra-cellular slime macromolecules, the products with or without NAC exposure under various conditions were subjected to cellulose acetate electrophoresis using a modification of various methods described in the literature (Bartlett 1963, Hronowski & Anastassiades 1980, Poulsen 1983, Scherr 1962, Wessler 1968).

Calgary biofilm device assay

To find the optimal synergistic concentrations required for eradicating biofilms using various concentration gradients of NAC and rifampicin/clindamycin the effects on mature biofilms were quantified using the Calgary biofilm device assay. This has recently been renamed the Minimum Biofilm Eradication Concentration (MBEC) assay. A modification of the method described by Harrison et al., (2010) was used in order to perform a checkerboard format to test synergy. The principles of synergy testing were obtained from Moody (2004) and were applied to the MBEC assay after modification.

Section 3. Bacterial Identification

3.1 Choice of bacteria

Choosing the bacteria was an important step of the project, as this may give misleading results if the bacteria do not resemble the ones found in the middle ear. Conventionally the commonest middle ear isolates in OME are *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae* (Giebink *et al.*, 1982, Stanievich *et al.*, 1981). More recently Paluch-Oles et al. (2011) demonstrated that coagulase negative staphylococci were the dominating organisms in 30.9% of cases of culture positive samples. The most common of those was *S epidermidis*. This confirms our findings from a study performed by the Biomaterials - Related Infection Group (BRIG) laboratory, Nottingham (Daniel *et al*., 2012 - b). Middle ear effusion samples were collected from children undergoing myringotomy and ventilation tube insertion for OME. Bacteria in biofilms are highly dormant in a slow growing state and short culture periods may result in apparently negative growth, therefore extended culture techniques on the samples were adopted to allow for this. *Coagulase negative staphylococci* (CoNS) were the most commonly cultured organism in 12.9% of effusions followed by *Veillonella spp* 9.7%, *S aureus* 8.1%, *S pneumoniae* 6.5%, *Bacillus spp* 4.8%, *M catarrhalis* 4.8%, and *Pseudomonas spp* 4.8%.

Conventionally CoNS have been regarded as contaminants due to their presence as normal skin commensals. Additionally, short incubation periods may not have been adequate for the growth of SCV's that have been discussed earlier in section 1.4.6. It is now widely accepted that CoNS and *S aureus* are major causes of biofilm - related infections and it is not surprising that they are the commonest middle ear isolates in the Daniel *et al*. (2012 - b) study. Bacterial isolates from that study were stored in the BRIG laboratory following identification and given individual catalogue (F) numbers. All bacterial samples were stored at -22 °C. For this study isolates were chosen from the same pool of bacteria and re-identified prior to further use. In order

to represent the common culprits of middle ear infection it was considered appropriate to use isolates based on the recent findings: *S aureus* and *S epidermidis*, in addition to the conventional isolates; *H influenzae, S pneumoniae,* and *M catarrhalis*. As the scope of the project became more focused and further experiments were needed to elucidate the mechanism of action of NAC on Extra-Cellular Slime (ECS), one bacterium that was a good biofilm producer was used to illustrate a proof of concept. This allowed for more experiments to be performed given the same time frame.

3.2 Methods

Unless otherwise stated culture medium was purchased from Oxoid Ltd, Basingstoke, UK and reagents were purchased from Sigma-Aldrich, Poole, UK. Sheep blood agar plates and chocolatised blood agar plates were used to grow bacteria, 5% CO^2 was used to grow *S pneumoniae*. To confirm bacterial identity a series of tests were performed; catalase test, gram staining, and microscopy. DNAse agar plates were used to differentiate *S aureus* from *S epidermidis* with a positive and negative control. To confirm identity API strips (bioMérieux SA, France) were used according to manufacturer's instructions. APIWEB software was used to analyse the results of the API strip. Optochin discs were used to confirm identity of *S pneumoniae*. X, V, and XV factor discs were used to confirm identity of *H influenzae*. Agar plates with *S pneumoniae* were incubated in 5% CO^2 at 37˚C.

Trypticase Soy Broth (TSB) growth medium was used for all bacteria. When growing *S pneumoniae*, TSB was supplemented with 5% defibrinated horse blood. When growing *H influenzae*, TSB was supplemented with β-NAD 30mg/L and haemin 30mg/L.

3.2.1 Optochin susceptibility test

S pneumoniae was grown overnight on sheep blood agar with an optochin disc placed in the inoculum area. Ethyl hydrocupreine hydrochloride (optochin) is able to inhibit the growth of *S pneumoniae* and will therefore differentiate pneumococci from other viridans streptococci. A positive test would show an area of inhibition around the disc.

3.2.2 X and V test

To confirm the identity of *H influenzae* an X & V factor test was used. *H influenzae* was grown on Tryptone Soy Agar (TSA) overnight with X, V, and XV discs placed on the plate separate from each other. *H influenzae* needs both X and V to grow and should therefore grow only around the XV disc for a positive test.

3.2.3 Minimum Inhibitory Concentration (MIC) determination using E-test strips

Fresh overnight culture of the desired bacteria on agar plate was suspended by picking two to three colonies and mixing with sterile water to make 0.5 McFarland (0.08 optical density-OD at 490nm wavelength). This was plated on ISA (Iso-Sensitest Agar) leaving a small island strip with no inoculum. An E-Test strip (bioMérieux SA, France) of the desired antibiotic was placed onto the island strip on the ISA plate and this was incubated overnight at 37˚C. The line between inhibition and growth was read corresponding to the MIC reading on the E-Test strip.

3.3 Results

Out of various bacterial strains tested from the pool, the following four strains were found to be suitable for further testing (Table 3.1):

F no.	Species	DNA'se	Haem-olysis	Oxidase	Catalase	Gram stain
2544	*S pneumoniae*	-ve	-ve	Not performed	-ve	+ve
2543	*M catarrhalis*	+ve	-ve	+ve	+ve	-ve
2405	*S aureus*	+ve	-ve	Not performed	+ve	+ve
2494	*S epidermidis*	-ve	α	Not performed	+ve	+ve

F no.	Species	Shape	API
2544	*S pneumoniae*	Diplo-cocci	None
2543	*M catarrhalis*	Cocci	0210 99.9% *Morax.*
2405	*S aureus*	Cocci grapes	6736153 97.8% *S aureus*
2494	*S epidermidis*	Cocci grapes	6606013 98% *S epi*

Table 3.1 Results of identity testing for bacteria found to be suitable for further use

Further testing of F2544 with the optochin disc was performed to confirm that it was *S pneumoniae*. All *H influenzae* strains from the bank were dead. Therefore a wild strain was obtained from the Nottingham University Hospital, Microbiology Laboratory, isolated from a child with discharging eye. The following results were obtained:

Catalase +ve, Oxidase +ve, X & V test (grew around XV tablet confirming *H influenzae*), Gram –ve, appearance under microscopy: small cocco-bacilli.

3.4 Comments

A number of bacteria in the bank were found to be dead. This was more common with *H influenzae* and *S pneumoniae*. These bacteria are known to require specific environmental conditions for their survival, especially with *Haemophilus* species. The initial bacteria were isolated from MEE fluid of OME children in 2006 and this study was started in September 2010, approximately 4 years of storage time. To reduce the likelihood of bacterial death when storing bacteria long-term, a colder environment is generally advised -70°C, and -22°C may not have been suitable. It was also found that many samples were contaminated with *Bacillus* species, and have been discarded; this may have been due to previous unsterile technique.

Section 4. Crystal Violet Biofilm Assay

Bacteria with strong biofilm - producing abilities were chosen for *in vitro* testing: F2494 (*S epidermidis*), F2405 (*S aureus*), F2543 (*M catarrhalis*), F2544 (*S pneumoniae*). The wild strain of *H influenzae* previously described in section 3.3 was used. A fast high-throughput technique, the 96 well plate method described by Stepanovic *et al.*, (2000) was used with minor modifications.

4.1 Methods

Two to three colonies of a fresh overnight subculture from the bacteria to be tested were taken from a culture plate and inoculated onto 5mL of TSB (supplemented when needed). This was left on an orbital incubator (Stuart Scientific S150 Orbital Incubator) shaking at 220rpm overnight at 37˚C to achieve a 0.6-0.7 McFarland standard. Serial dilutions of the inoculum were cultured to confirm the number of Colony Forming Units (CFU) for a 0.5-0.6 McFarland standard per bacterial strain. 200µL of the inoculum was pipetted into each well of a 96 well flat bottom clear plate (Sterilin Ltd, Cambridge, UK). Each bacterial strain was tested in triplicate. The plate was covered with a lid and placed in a humidified incubator for a desired period of time at 37˚C. After removal of the plate the contents of each well were aspirated with a vacuum suction machine (2511 Dry Vacuum Pump) connected to a glass micropipette. To rinse the wells they were filled with 200µL of phosphate buffered saline (PBS) after which they were aspirated. Rinsing was repeated twice.

To fix the biofilms on the base of the wells 150µL of methanol was added to each well. This was left for 20 minutes after which the plate was tilted upside down and flicked a number of times to ensure removal of the methanol. The plate was then placed in a drying cabinet for 10 minutes to dry. The oven did not have a temperature monitor and has since been replaced. An estimation of the temperature by the author was from 55 to 65 °C with the full appreciation that this may not be accurate. Despite this it was not deemed to be a critical measure because this assay

was not measuring live viable biofilm where temperature may affect viability. For staining, 150µL of 2% crystal violet was added to each well and left for 15 minutes. Following aspiration of the crystal violet, the plate was placed under gentle tap water and rinsed five times until the water looked colourless. The plate was allowed to dry in the drying cabinet for 30 minutes. 150µL of absolute ethanol (instead of glacial acetic acid originally described by Stepanovic *et al.*, [2000]) was added and left for 30mins and then aspirated. 100µL of each well was transferred to a new 96 well fresh clear plate and this was read at 630 nm wavelength using a microplate reader (Bio-Tek EL800 Absorbance Microplate Reader). The optical densities were recorded with standard deviations. KC4 Microtitre reader software was used to record results that produced an Excel spreadsheet.

S pneumoniae and M catarhalis were grown in TSB supplemented with 5% defibrinated sheep blood. *H influenzae* was grown in TSB supplemented with 30mg/L of haemin (X factor) and 30mg/L of β - NAD (V factor) alone and also in TSB supplemented with X and V and 5% defibrinated sheep blood.

4.1.1 Method development and modifications

To prevent washing away the biofilms the rinsing was reduced to one rinse with PBS. To catch and process the air-biofilm interface at the top of the growth medium meniscus the following have increased:
Fix with 200 µL methanol instead of 150 µL
Stain with 200 µL of crystal violet instead of 150 µL
Add 200 µL of ethanol instead of 150 µL

On further inquiry following completion of the assay the above step may have affected the results detrimentally as the meniscus level was not thought to represent biofilm and is generally avoided. Despite this, the above modification would have been repeated uniformly throughout the wells and therefore comparisons should theoretically still be valid. As the slough/slime layer for *H influenzae* and *S pneumoniae* at the bottom of the well was being washed away in the rinse step, tissue culture - treated 96 well flat bottom plates (Berthold Technologies Ltd,

Herpenden, UK) were used instead. *S pneumoniae* slime did not visibly wash away in the rinsing step however *H influenzae* still washed away.

4.1.2 Data analysis

Each bacterial strain was tested in triplicate and the result of the optical density representing biofilm quantity was presented as a mean of three OD readings. The background OD was subtracted from the mean score to give the final OD reading presented in table 4.1. The data was collected using Excel spreadsheet. Statistical analysis was not performed, as this was not applicable.

4.2 Results

The four bacterial strains from the OME bacteria pool (F2494, F2405, F2543, and F2544), and the wild strain of *H Influenzae* were used. Background growth medium controls were included. A weak biofilm producer (F19 - negative control, *S hominis*) and a strong biofilm producer (F20 - positive control, *S epidermidis*) were also included. The results shown below were the average of readings performed in triplicate. In order to quantify the biofilm, the background OD of the growth medium was subtracted from the OD of the individual bacterial biofilms. TSB gave an OD of 0.047, XV supplemented TSB gave 0.062, and blood - supplemented TSB gave 0.247.

XV:

TSB with XV was 0.062 & TSB alone was 0.047

∴ XV was calculated as 0.062 – 0.047 = 0.015

Blood:

TSB with blood was 0.247 & TSB alone was 0.047

∴ Blood alone was calculated as 0.247 – 0.047 = 0.2

The background was subtracted from the OD to obtain biofilm quantification for each bacterial species (Table 4.1).

Solution	Calculation	Optical Density (OD - A630nm)
F19 (*S hominis*)	0.084 - 0.047	**0.037**
F20 (*S epidermidis*)	0.109 - 0.047	**0.062**
F2494 (*S epidermidis*)	0.207 – 0.047	**0.160**
F2405 (*S aureus*)	0.082 – 0.047	**0.035**
F2543 (*M catarrhalis*)	0.374 – 0.247	**0.127**
F2544 (*S pneumoniae*)	0.426 – 0.247	**0.179**
Wild strain *H influenzae* with XV	0.059 – 0.062	**- 0.003**
Wild strain *H influenzae* with XV and Blood	0.131 – (0.015+0.2)	**- 0.084**

Table 4.1 Results of optical densities representing biofilm quantification for the various bacterial species using crystal violet assay.

4.3 Comments

The crystal violet biofilm assay was found to give highly variable results with minor changes to the methodology. It also gave variable readings if repeated with the same methodology. We have made many changes to the original methodology proposed by Christensen *et al*., 1985, to try to achieve consistent results, but failed to do so. One of the most important factors was thought to be the washing step. It was found that if the wells were washed three times, there would not be any biofilm left, and if it was washed once then there was an overestimation of biofilm. The other highly variable step was the removal of planktonic suspension prior to washing with PBS. The original proposed method to aspirate with a pipette gave varying readings when done in triplicate due to technique variation. This was standardised by using a vacuum suction to remove the planktonic suspension.

Overall this assay was not consistent therefore the findings needed to be read with caution. Taking that into consideration, it was useful to get an estimate of the biofilm -producing potential of the test bacteria. *H influenzae* was found to adhere poorly to plastic surfaces and was being washed off by the PBS very easily. Introducing the tissue culture- treated plates instead of the plain ones did not improve the adherence of *H influenzae*. The *in vivo* conditions where *H influenzae* can thrive may not be easily replicated *in vitro* and conditioning films may need to be used to fully replicate its biofilm - producing potential. What gave further concern about this assay was the small difference in biofilm quantification between the positive and negative controls. The positive control (F20) has been chosen from previous work that has shown it to be a very strong biofilm producer, but in this assay it gave a reading of 0.062, very close to the negative control of 0.037. There are a number of reasons that may account for this. The other bacteria used in this assay may be stronger biofilm producers than the positive control; F2544 (*S pneumoniae*) for example had the highest reading followed by F2494 (*S epidermidis*). Alternatively, it may be that the conditions in which the F20 positive control has been used previously favour its biofilm productivity when compared to this assay. Work with F20 (*S epidermidis*) in the BRIG laboratory has been catheter-related where a high flow system and large shear forces are present. The crystal violet assay is a static

system closer to middle ear conditions, albeit without the mucosal surface element that may prove to be a further important factor that needs to be taken into consideration.

It has to be noted that staphylococci are highly adaptable bacteria able to adjust biofilm production depending on the growth conditions and may account for the variations observed. Stepanovic has mentioned many of the problems encountered in this assay and introduced modifications with a standardised technique (Stepanovic *et al*., 2000). It was felt that despite those modifications the fundamental flaws lie in the basic principals of the assay such as the highly variable rinsing step and the technique for removing the liquid from the rinsed wells.

Section 5. Silicone disc biofilm assay; Proof of concept

5.1 Introduction

A robust and validated assay was needed to test the hypothesis that NAC enhanced biofilm eradication of antibiotics. The BRIG group have previously developed and verified a robust model to test biofilms (Daniel *et al*., 2012 - a) based on the plate count principle. The plate count principle is a more accurate method of measuring biofilm quantity (Section 1.4.7) as it measures colony counts of bacteria extracted directly from the biofilm using sonication, therefore having fewer variables that may introduce error. The biofilm model makes use of silicone discs 4mm in diameter cut out from silicone sheets onto which biofilms can grow on (silicone elastomer sheet thickness 1.0 mm, 150mmx150mm, Goodfellow Cambridge Ltd., Huntingdon, UK). The use of medical grade silicone was initially intended however silicone with a much more rough surface was ordered in error. It was recognised that the middle ear mucosa may have different properties that may not be reproducible in the laboratory, however in order to have proof of concept it was felt that the silicone discs would be used. The silicone discs were placed in bijoux bottles with 2 mL of growth medium, resembling the middle ear space that has an approximate volume of 2 mL. Anti-biofilm agents may be added to the 2mL volume for a desired period of time following which the discs can be removed for the biofilm to be quantified.

Due to the inconsistencies of the crystal violet assay in identifying biofilm-producing capacity, a *S aureus* strain previously used in this assay with proven biofilm-producing capacity was used to test the efficacy of NAC. The advantage of using the same bacterial strain and the same biofilm model was that the results from this work and the previous work could be compared. Previously, Daniel *et al*. (2012 - a) tested rifampicin and clindamycin in combination at 100 and 1000 MIC against 5-day-old mature biofilms. Biofilms were eradicated after exposure to rifampicin and

clindamycin used together at a 1000 MIC for two weeks. This is a very high antibiotic concentration, and it was hoped that the addition of NAC would enhance this eradication thus reducing the concentration of antibiotic needed. To get proof of concept we used NAC at a high concentration of 50 mg/L. If there were an enhancement in biofilm eradication then the MBEC 96 well plate high-throughput assay would be used to find the optimal concentration of NAC needed.

5.2 Methods

The *S aureus* F2315 obtained from the OME bacteria pool was tested to confirm identity (Tabe 5.1).

F number	Species	DNA'se	Haemolysis	Oxidase	Catalase
2315	*S aureus*	+ve	-ve	-ve	+ve

Gram stain	Shape	API	Antibiogram
+ve	Cocci grapes	6336143 92% *S.aureus*	40000

Table 5.1 Result of identity testing for *S aureus* F2315 prior to further use in silicone disc biofilm assay.

Initially the MIC value of F2315 strain was determined using E-test strips as described in section 3.1.4 for rifampicin and clindamycin. Rifampicin MIC was 0.002 mg/L, and clindamycin MIC was 0.094 mg/L.

5.2.1 Making the biofilms

Fresh F2315 was cultured on sheep blood agar plate overnight. A 0.5 McFarland standard (0.08-0.13 optical density at 490nm) was made by picking up two to three colonies with an inoculation loop and suspending in TSB. The inoculum CFU's was worked out separately by serially diluting a sample of the original suspension and plating onto sheep blood agar. Silicone discs were sterilised by autoclaving and suspended in the TSB bacterial suspension in a 30 mL universal bottle. This was then incubated in an orbital shaker at 37˚C with 150 rpm for 1 hour and then still for one hour to allow the bacteria in suspension to attach to the silicone discs. Clean control discs were also used to check for any contamination in methodology. Sterile Bijoux bottles were set up so that each bottle contained 2 mL of sterile TSB. The

discs were rinsed three times in PBS to wash off any loose bacteria and a silicone disc was transferred into each of the bottles containing TSB. To test for planktonic bacteria 20 µL of the original inoculum suspension was transferred to separate Bijoux bottles with 2 mL of TSB without any silicone discs.

Following preparation of the above bottles these were incubated at 37˚C for 5 days to allow biofilms to develop and mature on the silicone discs. After the 5 days the treatment was added to the bottles depending on the treatment arm. The assay was planned so that there were 6 arms of treatment:

1 - No Treatment
2 - 100 MIC
3 - 1000 MIC
4 - NAC 50 mg/mL
5 - 100 MIC + NAC 50 mg/mL
6 - 1000 MIC + NAC 50 mg/mL

Note: 100 MIC means 100MIC of rifampicin + 100MIC of clindamycin. This also applied to 1000MIC. There were clean controls in each of the 6 arms with each performed in triplicate. Each arm tested planktonic bacteria and bacteria in biofilm. There was a spare disc for each treatment arm in case of any errors or contamination.

5.2.2 Making the anti-biofilm agents

MIC for F2315 of clindamycin was 0.094 mg/L (µg/mL), and of rifampicin was between 0.002 to 0.003 mg/L (µg/mL). CFU's of planktonic suspension used to perform MIC was confirmed by overnight plating onto sheep blood agar plate. This gave 4 x 10^8 CFU's/mL. The calculations were made to give a concentration of either 100 MIC or 1000 MIC of antibiotics into a fixed volume of 2 mL.

5.2.3 Rifampicin

Base solution was made to a concentration of 30 mg/mL ie. 30 μg/μL making 10000 MIC, then calculations were performed to work out the dilutions required for the specific MIC values (Table 5.2).

MIC	Concentration	Label	μg/mL	μg/2mL	Dilution method
1	Low	L	0.003	0.006	2μL of 1:10000 of base into 2mL
10	Medium Low	ML	0.03	0.06	2μL of 1:1000 of base into 2mL
100	Medium High	MH	0.3	0.6	2μL of 1:100 of base into 2mL
1000	High	H	3	6	2μL of 1:10 of base into 2mL
10000	Very High	VH	30	60	2μL of base solution into 2mL

Table 5.2 Calculations performed for the dilutions necessary to obtain *S aureus* F2315 MIC values for rifampicin.

1000 MIC: Base solution diluted 1:10 with sterile H_2O, 2 μL added to each bijoux bottle containing 2 mL of TSB giving total concentration of 3 μg/mL ie. 1000 MIC.

100 MIC: Base solution diluted by 1:100, then 2 μL added to each bijoux bottle containing 2 mL of TSB giving total concentration of 0.3 μg/mL ie. 100 MIC.

5.2.4 Clindamycin

Similarly, base solution was made to a concentration of 100 mg/mL ie. 100 µg/µL making 10000 MIC, after which the following serial concentrations were made by serial dilutions corresponding to the given MIC's (Table 5.3):

MIC	Concentration	Label	µg/mL	µg/2mL	Dilution method
1	Low	L	0.1	0.2	2µL of 1:10000 of base into 2mL
10	Medium Low	ML	1	2	2µL of 1:1000 of base into 2mL
100	Medium High	MH	10	20	2µL of 1:100 of base into 2mL
1000	High	H	100	200	2µL of 1:10 of base into 2mL
10000	Very High	VH	1000	2000	2µL of base into 2mL

Table 5.3 Calculations performed for the dilutions necessary to obtain *S aureus* F2315 MIC values for clindamycin.

1000 MIC: Base solution diluted 1:10 with sterile H_2O, 2 µL added to each bijoux bottle containing 2 mL of TSB giving total concentration of 100 µg/mL ie. 1000 MIC.

100 MIC: Base solution diluted by 1:100, then 2 µL added to each bijoux bottle containing 2 mL of TSB giving total concentration of 10 µg/mL ie. 100 MIC.

5.2.5 Combining rifampicin and clindamycin

Rifampicin and clindamycin were tested in combination and not separately in all the experiments. Due to this a final total volume of 2 mL contained 3 µg/mL of rifampicin and 100 µg/mL of clindamycin for a 1000 MIC solution, and 0.3 µg/mL of rifampicin and 10 µg/mL of clindamycin for a 100 MIC solution.

5.2.6 NAC

The target concentration needed in 2 mL of each bijoux bottle was 50 gm/L. 10 gm of NAC was added to 10 mL of de-ionised H_20 (dH_2O), then heated to 85°C in a hot bath for the crystals to dissolve to make 10 gm/10mL or 10000 mg / 10000 µL. To calculate how much volume was needed for 100 mg, the following calculation was used:

10000 mg → 10000 µL

100 mg → x

x = (100 mg X 10000 µL) / 10000 mg = 100 µL (ie. 100 µL will contain 100mg)

∴ Adding 100 µL of NAC solution to each bijoux bottle containing 2 mL of TSB gave an NAC concentration of 50 mg/mL or 50 gm/L.

The data collection template used for inputting the results of the silicone disc biofilm assay was as shown Table 5.4. The template also shows the general layout of the assay with each of the tested combinations.

Treatment regimen	sampling day	Planktonic	Biofilm disc 1	Biofilm disc 2	Biofilm disc 3	Clean disc
No Rx	1					
	7					
	resus					
	14					
	resus					
	spare					
100MIC	1					
	7					
	resus					
	14					
	resus					
	spare					
1000MIC	1					
	7					
	resus					
	14					
	resus					
	spare					
NAC	1					
	7					
	resus					
	14					
	resus					
	spare					
100MIC	1					
NAC	7					
	resus					
	14					
	resus					
	spare					
1000MIC	1					
NAC	7					
	resus					
	14					
	resus					
	spare					

Table 5.4 Data collection template of silicone disc biofilm assay. Each arm was performed in triplicate with a clean control and a planktonic suspension. Biofilms were tested against rifampicin/clindamycin combination at concentrations 100 and 1000 MIC with and without NAC. A growth control arm with no NAC or antibiotics was prepared. Resuscitation discs (resus) were prepared in case there was no growth to confirm whether biofilms were inhibited or eradicated (see Section 5.2.9).

5.2.7 Biofilm sampling

After the desired periods of anti-biofilm exposure the discs were transferred to sterile eppendorfs using sterile forceps. The forceps were sterilised in between discs by dipping into absolute ethanol then flaming in a Bunsen burner then cooling in sterile PBS. Each sample was washed in PBS three times. 500uL of trypsin was added to each sample, and incubated at 37°C for 15 min. Trypsin was aspirated from the eppendorfs and 1 mL of PBS was added to each sample and placed in the sonicator. Water was added until it covered the legs of the holder, and was just level with the bottom of the eppendorf holding platform. Sonication was for 5 min with temperature set at 22°C and a frequency of 50Hz (Ultrawave limited, Cardiff, UK). The liquid was plated after sonication undiluted (neat) and at 10^{-3} and 10^{-6} dilutions for the actual biofilms. 10^{-3} and 10^{-6} were plated for the no-antibiotics controls. The clean discs were plated neat. Fresh PBS was used for dilutions. Plates were incubated at 37°C and CFUs were read after both 24 and 48 hours of incubation.

5.2.8 Planktonic solvent sampling

For the bijoux bottles that do not contain a biofilm disc, planktonic bacterial growth was measured after the desired period of anti-biofilm exposure using serial dilutions. A 200µL sample of each bijoux bottle was plated at neat, 10^{-3} and 10^{-6} dilutions. The plates were incubated at 37°C and CFU's were read after both 24 and 48 hours incubation.

5.2.9 Resuscitation

The resuscitation discs of the treatment arm that did not show growth was processed as follows to see whether biofilms were inhibited or eradicated: the TSB / anti-biofilm mixture was removed from the bijoux and the discs were rinsed 3 times with PBS. Fresh TSB was placed into the bijoux and incubated for 5 days before sampling as above.

5.2.10 Bacterial identification

To check for contamination or development of resistance and to confirm bacterial identity, samples were chosen at the beginning of the assay and at the end of the assay (after resuscitation). The samples were run through a battery of tests; MIC, API, DNA'se, Oxidase, gram staining, colony morphology – micro and macroscopic appearance, and anti-biogram.

5.2.11 Data analysis

There were three biofilm discs prepared for each treatment arm. This was depicted in the data collection template (Table 5.4). One planktonic bottle and one clean disc control were also prepared for each treatment arm. Following processing of the three biofilm discs for each treatment arm, the CFU counts were inserted into an excel spreadsheet. Each of the CFU counts was converted to the log CFU count. The mean of the three log CFU counts was represented in the graphs (Figure 5.1 to 5.3). As the aim was to eradicate biofilm with a CFU count of zero, statistical analysis was not applicable.

5.3 Results

5.3.1 One day exposure

After five – day old mature biofilms were exposed to anti-biofilm agents for one day, the results were as follows (Figure 5.1):

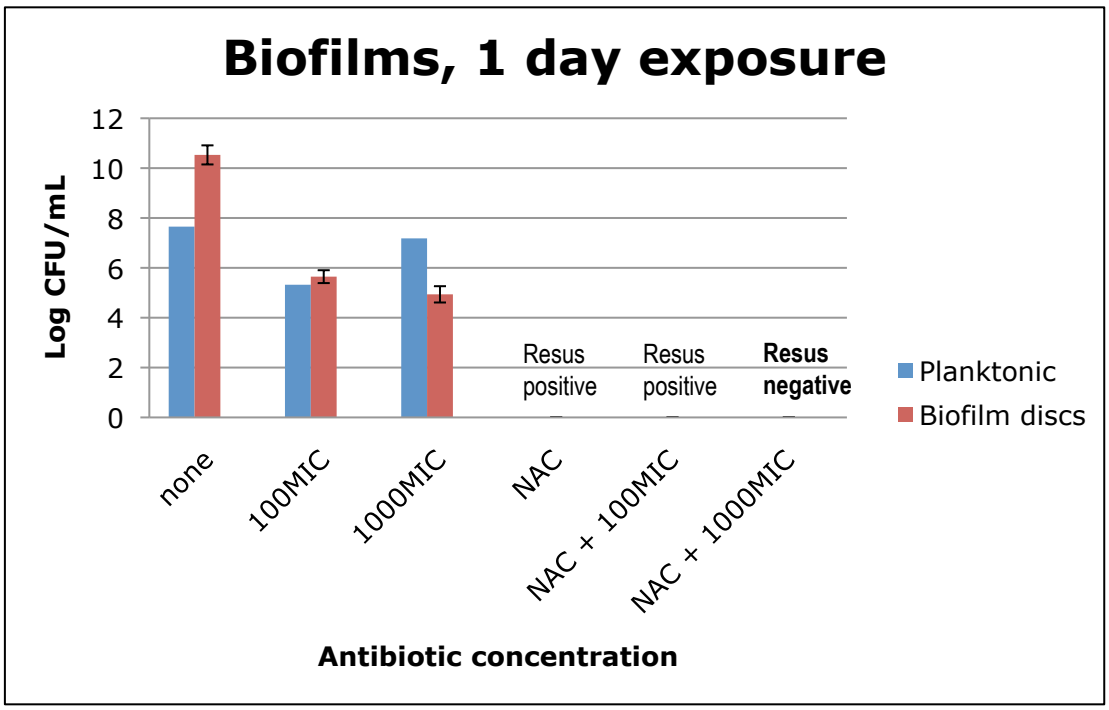


Figure 5.1 Silicone disc assay results from one-day exposure to combinations of NAC, and rifampicin with clindamycin together at 100MIC and 1000MIC. Resus positive: bacterial inhibition showing no growth, but on re-incubation and resuscitation bacteria grew. Resus negative: bacterial eradication with no growth following resuscitation.

The results of day one exposure showed that NAC with 1000MIC of antibiotics eradicated bacteria after twenty-four hours of exposure. NAC alone and with 100MIC of antibiotics resulted in inhibition of bacteria; they did not grow on culture, but when the discs were resuscitated, they grew again. This indicates that the bacteria were in a state of inhibition, a characteristic of biofilm bacteria. The resuscitation process was performed as per Section 5.2.9. Neither planktonic bacteria

nor biofilms were eradicated by antibiotics alone. Antibiotics alone resulted in a reduction of biofilm quantity to just under half the growth control.

5.3.2 Seven day exposure

After five – day old mature biofilms were exposed to anti-biofilm agents for seven days, the results were as follows:

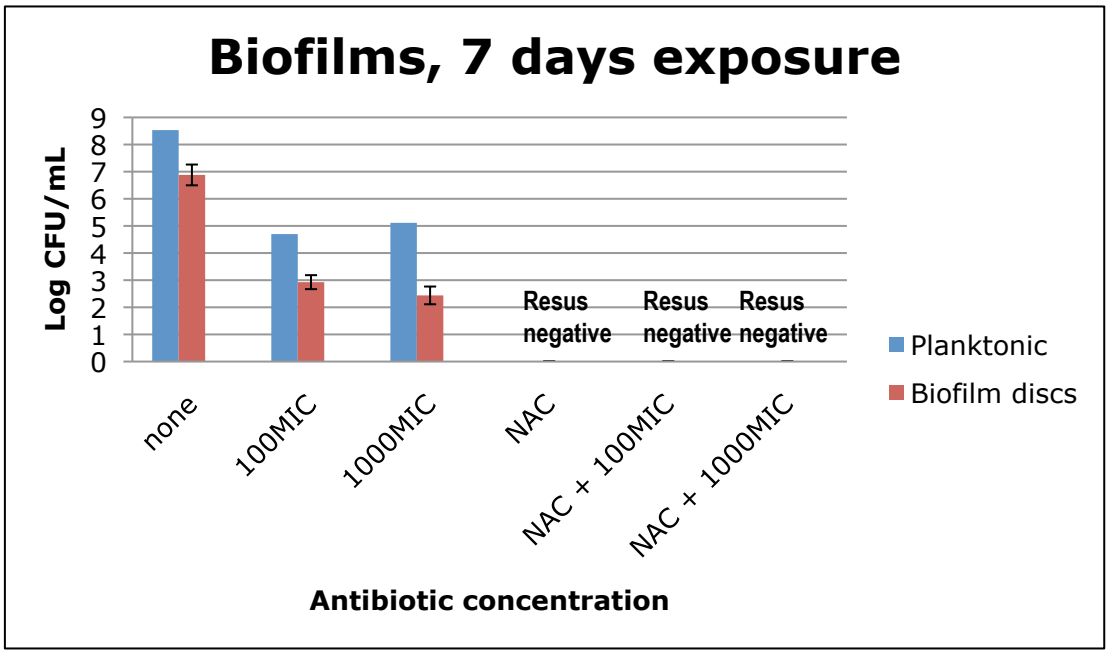


Figure 5.2 Silicone disc assay results from seven-day exposure to combinations of NAC, and rifampicin with clindamycin together at 100MIC and 1000MIC. Resus negative: bacterial eradication with no growth following resuscitation.

All biofilms were eradicated by NAC, with and without antibiotics. The resus discs corresponding to the concentrations showing no growth were re-incubated as per resuscitation protocol in Section 5.2.9 and there was no growth indicating eradication as opposed to inhibition. Antibiotics alone resulted in a reduction of biofilm quantity to just over half the growth control.

5.3.3 Fourteen days exposure

After five – day old mature biofilms were exposed to anti-biofilm agents for fourteen days, the results were as follows:

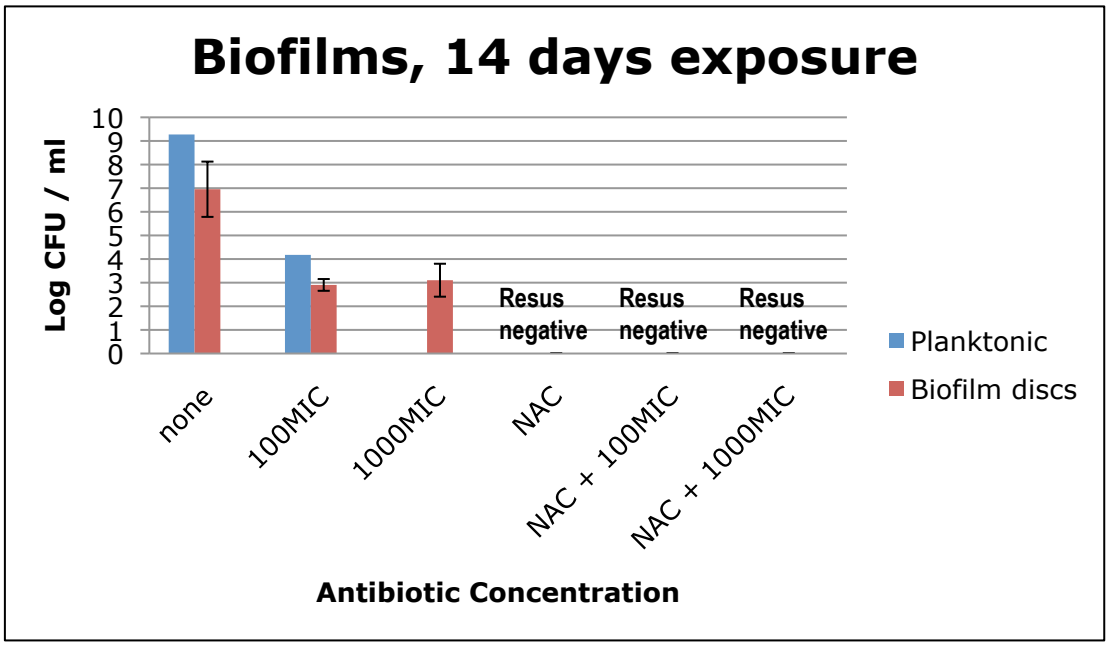


Figure 5.3 Silicone disc assay results from fourteen-day exposure to combinations of NAC, and rifampicin with clindamycin together at 100MIC and 1000MIC. Resus negative: bacterial eradication with no growth following resuscitation.

Antibiotics at 1000MIC eradicated planktonic bacteria, but failed to inhibit or eradicate biofilm. All biofilms were eradicated by NAC, with and without antibiotics. The resus discs corresponding to the concentrations showing no growth were re-incubated as per resuscitation protocol in Section 5.2.9 and there was no growth indicating eradication as opposed to inhibition.

5.4 Comments

The greatest benefit was derived when NAC was used in combination with 1000MIC, eradicating biofilms after just twenty-four hours exposure. Interestingly, NAC on its own after twenty-four hours inhibited biofilms, that is no bacteria was grown following processing of the biofilm disc unless the corresponding discs assigned for the resuscitation process were rinsed with PBS and re-incubated in TSB for five days after which growth was observed. NAC on its own eradicated biofilm after seven days and this calls into question whether antibiotics are needed at all. These results indicate a very potent effect of NAC raising concerns over its safety for use in the middle ear and prompting an examination into the underlying mechanism of action. This is discussed in greater detail in the next section.

Antibiotics on their own failed to eradicate bacterial biofilms at the highest concentration tested, 1000MIC, and for the longest period tested of fourteen days, but when compared to their efficacy against planktonic bacteria these were at the 1000MIC after fourteen days' exposure. This is not surprising given the benefits bacteria incur when existing in a biofilm state. This supports the various explanations of this recalcitrance proposed in Section 1.4.6. Given the results it may have been beneficial to have further treatment arms extending to twenty-one days or even longer if practically possible to see how long it takes for each treatment arm to inhibit or eradicate bacterial biofilm. This would have implications to the design of the middle ear drug delivery pellet. Lower concentrations of anti-biofilm agents released for longer periods of time may be used to achieve the same goal of eradicating biofilms. It is unknown however which is more ototoxic: a low concentration of a drug released over a prolonged period of time or a burst of high concentrations in a short period of time. Toxicity of aminoglycosides is closely dependent on intracellular concentrations, although neither rifampicin nor clindamycin are aminoglycosides toxicity may similarly be related to intracellular concentrations. The author therefore feels that a burst of high concentration in a short period of time may achieve higher intracellular concentration levels posing a higher risk of toxicity but in vivo toxicity studies are needed to answer these questions.

It would also have been interesting to compare the effects of anti-biofilm agents on biofilms in various stages of maturity. Mature biofilms are known to be much harder to eradicate, but it may not be a linear relationship. A study examining the susceptibility of *Burkholderia cepacia* to ciprofloxacin and ceftazidime showed that biofilm bacteria increased their recalcitrance to ciprofloxacin by a factor of 150 during the exponential phase until reaching stationary phase (Desai *et al*., 1998). Similarly, biofilm bacteria at stationary phase were 80 times less susceptible to ceftazidime than biofilm bacteria at growth phase, nine generations before reaching stationary phase (Desai *et al*., 1998). This has important clinical implications when treating biofilm-related infections where the treatment regimen needs to be tailored to the degree of maturity of the biofilms involved. Practically however it is difficult to measure biofilm maturity without expensive imaging or labour - intensive biofilm assays and it is better to assume that biofilms have reached stationary phase growth. This is an important reason to consider the use of biofilm - reducing agents such as NAC in conjunction with antibiotics to ensure eradication of bacteria. Before NAC can be safely used in the middle ear it is important to explain the potent effects observed in this assay, and this is explained in Section 6.

Section 6. Mechanism of action of NAC

6.1 Introduction

The potent action of NAC observed in the silicone disc biofilm assay raised the possibility of a pH - dependent effect. An acidic environment is known to be bactericidal to many bacteria. The pH of NAC was measured and found to be low at various concentrations (Table 6.1). This questions whether the findings observed were purely pH-related or whether there was a combination of NAC-related effect together with that of an acidic environment.

Concentration	pH in d-H_2O	pH in TSB
100gm/L	1.89	2.37
10gm/L	2.35	3.72
1gm/L	2.81	6.80
0.1gm/L	5.03	7.13
0.01gm/L	6.55	7.21
0.001gm/L	6.70	-----
de-ionised H_2O	6.60	-----

Table 6.1 pH Table of NAC solution using de-ionised H_2O and TSB as the solvent.

The first question that needs to be answered is whether NAC and pH are bactericidal against planktonic bacteria. The MIC of NAC in broth culture was therefore determined with both pH adjustment and no adjustment for *S aureus* in suspension. More importantly, the effects of NAC and/or pH on the biofilm matrix needed to be elucidated. The ultimate goal is to break down the biofilm matrix to free bacteria into the free-floating form thus rendering bacteria defenceless from the protective effects of being in a biofilm state. To investigate this, a method was needed to extract extra-cellular slime (ECS) and to test against NAC both with and without pH adjustment. ECS refers to the tough polysaccharide matrix of the biofilm. An assay for the production of ECS was adopted from Bayston & Rogers (1990) where a series of steps have been described to purify polysaccharide intercellular adhesin (PIA), the

main ECS of *S aureus*. Various combinations of NAC both pH and no pH adjustment were added to ECS following which the products were analysed through two further assays.

The first of the assays quantifies glycosaminoglycan (GAG) using alcian blue dye. Extracellular slime produced by bacteria consists of large GAG molecules. If NAC breaks down ECS then in theory a reduction in the total GAG quantity is expected. A few papers in the literature have described the quantification of GAG molecules using the principle that alcian blue dye attaches to the GAG complexes, after which the amount of dye attached can be measured spectro-photometrically to give an indication to the amount of GAG in solution. The method used in this project was based on that of Bayston and Rogers (1990). In addition two other papers describing the basic principles were also used as references (Whiteman 1973, Burlingame *et al*., 1981).

The GAG alcian blue assay does not give an indication of the size of the molecules and therefore may not determine whether NAC breaks GAG molecules. The total GAG quantity would remain the same if the molecule were broken in half thus a major change in the basic structure and size of the GAG molecule is not detectable. For this reason cellulose acetate electrophoresis is used concurrently alongside the GAG alcian blue assay. Electrophoresis is based on the principle that molecules travel at various speeds through a medium under an electric current, depending on the size of molecule. The choice of medium is dependent on the size of the molecules to be examined. SDS-gel medium is suitable for proteins, RNA, and DNA, but GAG's are much larger molecules. Cellulose acetate paper has large pores allowing the movement of large GAG molecules and is therefore the medium of choice. Cellulose acetate electrophoresis is used to diagnose patients with mucopolysaccharidoses by examining the urine GAG's. The difficulty with cellulose acetate electrophoresis is that there is no standardised method making it difficult to compare the results of papers. A number of studies describing this assay have been identified, each with small variations in the methodology (Bartlett 1963, Hronowski & Anastassiades 1980, Poulsen 1983, Scherr 1962, Wessler 1968). Our method adopted a combination of those protocols and refined for use to examine the size of ECS molecules with and without the addition of NAC.

6.2 Production of ECS

Two to three colonies of fresh subcultures of F2315 were inoculated into 15mL of TSB and vortexed. The suspension was incubated on an orbital shaker for 18 to 20 hours at 37°C and 200rpm speed to allow the replication of bacteria. The suspension was centrifuged at 3000rpm and 4°C, following which the supernatant was aspirated to remove any TSB and by-products of bacterial metabolism. Further washing and centrifugation was performed twice in sterile de-ionised water (centrifuging each time) then 10mL of sterile de-ionised water containing 20mg/L MgCl was added (MgCl hexahydrate - molar mass 203.31g/mol). The purpose of adding MgCl was to minimise the release of teichoic acids into the water, as these also react with alcian blue. The suspension then was vortexed, and 200µL was withdrawn and the A490nm recorded to determine bacterial quantity.

The washed bacteria in MgCl were incubated statically at 37°C for 18-20 hours to allow stationary phase development due to the absence of nutrient broth, thus allowing the production of ECS. To remove bacteria, the suspension was centrifuged at 3000 rpm at 4°C for 30 minutes and the supernatant containing the ECS was retained and stored at -22°C for further experiments.

Method development and modifications:

Two to three colonies were mixed with 20mL of TSB instead of 15mL to produce a larger quantity of ECS per assay run. Higher speeds of centrifugation were performed to remove bacteria from the ECS at 7500rpm for 15 minutes in 4°C as the solution was found to be slightly turbid at 3000rpm. The ECS produced was filter - sterilised to ensure freedom from bacteria. This filtration process was found not to have an effect on the final total quantity of ECS and was therefore performed routinely.

Following the above steps and with the modifications mentioned, ECS was stored in 20mL vials at -22°C for further testing with NAC.

6.3 Making neutralised NAC solution

(See Section 5.2.6 for making non-pH adjusted NAC)

Making stock solution of neutralised NAC at 100gm/L:

2gm NAC mixed in 8mL of deionised H_2O – pH 1.6 (Heating was performed using glass beaker over a Bunsen burner for approximately 30 seconds until crystals dissolved. Specific temperature was not measured and solution did not reach boiling point)

7.5 mL of NaOH (4M) + 0.5mL of deionised H_2O – total volume 16mL

2gm in 16mL = 1gm in 8mL = 0.125gm/mL = 125mg/mL

4 mL of deionised H_2O added to make total volume of 20mL

2gm in 20mL = 1gm / 10mL = 0.1gm / mL = 100 gm/L FINAL CONCENTRATION

Solution was filter - sterilised prior to using in assays

Making neutralised NAC at a concentration of 500gm/L (0.5gm/mL):

(Safety precautions were taken at this step due to highly molar concentration of NaOH)

Neutralised NAC at 100gm/L was the highest concentration achievable using NaOH (4M) solution to neutralise, due to the diluting effect of adding volume to neutralise. In order to achieve higher concentrations of NAC, NaOH pellets were dissolved in 2mL of deionised H_2O with heating to achieve higher molarity until saturation occurred and no further NaOH pellets were dissolvable. Minute volumes of NaOH were added to NAC at the desired concentration of 0.5gm/mL with minimum dilution and large effects on pH. This was titrated while measuring pH repeatedly until neutralisation was achieved to the desired pH. Solution was filter - sterilised prior to use in further assays.

6.4 Calculating the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of NAC

Non-pH adjusted NAC:

To calculate the MIC and MBC of NAC a broth serial dilution method was used. Using fresh subculture of *S aureus* (F2315), a 0.5 McFarland suspension was made. Non-pH - adjusted NAC solution at various volumes from a stock solution of 0.5gm/mL were added to bijou bottles and topped with TSB and 200µL of bacterial suspension to make a total volume of 2mL. Bijou bottles were incubated at 37°C overnight. The turbidity of the liquid was observed and documented; a clear suspension indicated inhibition of bacterial growth and a turbid solution meant that there was bacterial growth. To calculate the MBC, 200µL of each suspension was spread on a sheep blood agar plate and incubated for 24 hours to check for bacterial growth. No growth of colonies meant NAC was bactericidal at that concentration.

NAC concentration (mg/mL)	Visual turbidity after overnight incubation	Colony growth on sheep blood agar plate
70	Clear	Nil
65	Clear	Nil
60	Clear	Nil
55	Clear	Nil
50	Clear	Nil
45	Clear	Nil
40	Clear	Nil
35	Clear	Nil
30	Clear	Nil
25	Clear	Nil
20	Clear	Nil
15 (MBC)	**Clear**	**Nil**
10	Clear	Growth
9 (MIC)	**Clear**	**Growth**
8	Turbid	Growth
7	Turbid	Growth
6	Turbid	Growth
5	Turbid	Growth

Table 6.2 Result of broth serial dilutions of non-pH adjusted NAC with corresponding growth on agar plate to calculate MIC and MBC for *S aureus* (F2315).

The MBC was between 10 and 15mg/mL and MIC was 9mg/mL for non-pH adjusted NAC (Table 6.2). It was deemed unnecessary to count CFUs for the NAC concentrations that did show growth (5mg/mL to 10mg/mL) because there was a clear line at 15mg/mL where there was no growth of the undiluted sample.

Neutralised NAC:

Neutralised NAC from the stock solution of 0.5gm/mL was used as per Section 6.2.2. Similarly various concentrations of NAC were made up this time from 10mg/mL to as high as 200mg/mL. This was in anticipation that higher concentrations of NAC were needed to achieve similar MIC and MBC values due to the removal of the pH effect. In case the MBC was higher than the tested concentrations, serial dilutions of each NAC concentration was performed and plated on agar in order to count the CFUs. The log CFU counts were drawn on a graph to obtain an estimate of the MBC.

NAC concentration (mg/mL)	Visual turbidity after overnight incubation	OD (A490nm)	CFU / mL
Clear TSB	Clear	0.131	-
-	-	-	-
200	Light turbidity	0.108	6.5×10^6
190	Light turbidity	0.113	7×10^6
180	Turbid	0.134	6.5×10^6
170	Turbid	0.158	1.05×10^7
160	Turbid	0.194	2.5×10^7
150	Turbid	0.213	2.15×10^7
140	Turbid	0.243	3.5×10^7
130	Turbid	0.29	5.5×10^7
120	Turbid	0.297	4.5×10^7
110	Turbid	0.35	5.5×10^7
100	Turbid	0.407	7×10^7
90	Turbid	0.43	1.15×10^8
80	Turbid	0.464	1.35×10^8
70	Turbid	0.506	1.4×10^8
60	Turbid	0.586	2.5×10^8
50	Turbid	0.6	4.5×10^8
40	Turbid	0.635	7×10^8
30	Turbid	0.651	4.5×10^8
20	Turbid	0.64	5×10^8
10	Turbid	0.626	6×10^8

Table 6.3 Result of broth serial dilutions of neutral NAC with corresponding optical densities (OD 490nm) and CFU counts to calculate MIC and MBC for *S aureus* (F2315). MBC was not reached at the tested concentrations of NAC with 6.5×10^6 CFU/mL growth at 200mg/mL of neutral NAC. None of the NAC concentrations resulted in a clear solvent therefore the MIC was not reached.

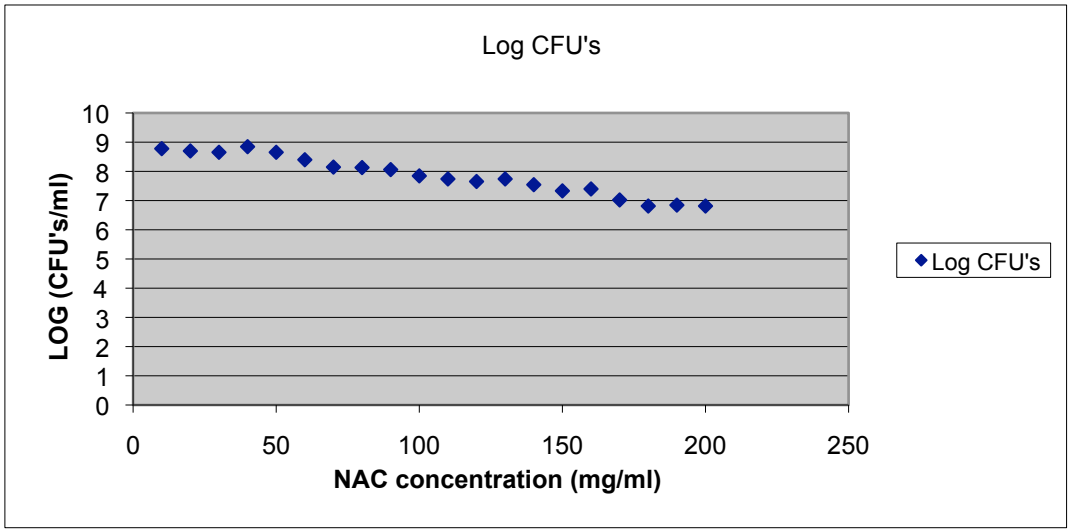


Figure 6.1 Results of log CFU counts following exposure to neutralised NAC at serial dilutions 10 to 200mg/mL. Extrapolation of the line would give an MBC that is unrealistically high.

The MIC of neutralised NAC was not achieved as all the solvents were turbid and the MBC was not found (>200mg/mL) because there was profuse colony growth in all sheep blood agar plates from the various concentrations (Table 6.3). An estimate of the MBC was not performed as can be appreciated from the graph because the NAC concentration would be unrealistically high if the extrapolated line were to meet the log CFU of 0 (Figure 6.1).

In summary, MIC of neutralised NAC was not achieved even at 200mg/mL neutral NAC compared to MIC of non-pH adjusted NAC of 9mg/mL. This indicates that the majority of the effect of NAC on bacteria in planktonic suspension was due to low pH. NAC may be bactericidal at a low pH, and bacterial death might not be secondary only to low pH. To investigate this, the MIC of non-pH - adjusted NAC needs to be compared with the MIC of an acid with similarly low pH. This was outside the remit of this project and was not performed. Although the effect of NAC on planktonic suspension was helpful to understand, more importantly the effect on biofilm matrix and its constituents was more relevant for this project; antibiotics were used for their bactericidal effect and NAC for the biofilm - reducing potential in addition to its mucus - reducing properties in the middle ear. ECS is the major constituent of biofilm and the direct effects of NAC on ECS in acidic and neutral environment need to be investigated, and this is explored in the following sections.

6.5 pH related solubility of ECS

An interesting observation was seen on addition of non-pH adjusted NAC to purified ECS at room temperature. NAC (50mg/mL) at pH of 1.4 caused instant turbidity of purified ECS. There were two possible reasons that needed to be investigated for the turbidity:

1 –NAC breaks the ECS large soluble molecules into smaller insoluble molecules that cause turbidity

2 – NAC causes aggregation of the intact ECS molecules causing them to become insoluble, without breaking the molecules into smaller structures

The turbid mixture was placed in a centrifuge and spun at 4000 rpm so that the insoluble particles fell to the bottom and formed a pellet. The pellet was removed from the rest of the mixture and examined by microscopy - no bacteria were seen, and there was clumped aggregate that was gram positive. In addition, no growth was observed when the pellet was spread on sheep blood agar plate and incubated at 37°C for 48 hours. This was not unexpected because ECS was purified from bacteria, and the low pH NAC would have killed bacteria any residual bacteria.

In order to see if the turbidity effect was due to NAC or low pH, hydrochloric acid was titrated to purified ECS until the a pH of 1.4 was reached. The mixture similarly turned turbid, and on reversal of the pH to neutral by titrating with NaOH, the turbidity disappeared into a clear solution. This implied that ECS was insoluble at low pH possibly due to a change in the shape of the molecule due to electrostatic forces acting at a molecular level. Based on this simple observation it was not possible to conclude whether there was a reduction in the quantity of ECS or a breakdown in the molecule and therefore the further assays were also performed (Section 6.5, 6.6, & 6.7). Cellulose acetate electrophoresis would show if the ECS molecules had reduced in size. The total quantity of ECS was also measurable using the alcian blue GAG assay.

6.6 Alcian blue glycosaminoglycan (GAG) quantification assay

Alcian blue-tetrakis (methyl-pyridinium) chloride was obtained from Sigma-Aldrich (CAS no.: 123439-83-8, product no.: A4045). Alcian blue powder was dissolved in sodium acetate/magnesium chloride solution containing 50mM of each, and made to a pH of 5.8 to give a concentration of 50mg/100mL. One mL of the solution to be examined (example; ECS +/- NAC) was mixed with 4mL of alcian blue solution and left to stand for one hour at room to allow attachment of the alcian blue to the GAG complexes. The solution was centrifuged at 2800 rpm at 4 degrees for 20 minutes. The supernatant was gently aspirated, discarded and replaced by 4mL of absolute ethanol. After mixing with ethanol to wash away any unattached alcian blue, this was centrifuged at 2800 rpm and the supernatant aspirated, discarded and replaced with 4 mL of sodium dodecyl sulphate - SDS (100gm/L in 50mM sodium acetate, pH 5.8). The mixture was vortexed to dissolve the precipitate and this was then read spectro-photometrically. The readings were taken by aspirating 200μL of the SDS/alcian blue solution and mixing with 1mL of water and read at a wavelength of 620nm.

A negative and a positive control were performed. The negative background control was 1mL of de-ionised water added to 4mL of alcian blue and processed as above. The positive control was 1mL of chondroitin sulphate (25mg/L) added to 4 mL of alcian blue solution and processed as above. Chondroitin sulphate (CS) from shark fin was used (1238h Na Salt ex Shark, pure: Koch-Light Laboratories, Suffolk, UK).

Method development and modifications:

SDS needed to be heated in a water bath to 70°C to avoid crystallisation and allow better dissolution of the alcian blue precipitate. NAC was added to the negative and positive controls where 1mL of 50mg/mL of NAC was added to 4mL of CS solution. Alcian blue solution was found to precipitate on storage, adding to unwanted high background readings, and to counteract this, the solution was centrifuged prior to use to get rid of precipitate. Alcian blue solution was left at room temperature with the solution to be investigated for 2 hours rather than 1 hour to ensure attachment of all GAG's to the alcian blue dye.

To account for possible growth of bacteria and contamination in the purified ECS solution from the ECS assay, purified ECS was filter - sterilised prior to running the alcian blue assay. In addition, sodium azide was added to ECS at a concentration of 1% (1mg/mL). ECS was measured both with and without sodium azide to see if there were any bacteria in solution that may have accounted for a high reading.

Data analysis

On the first run of GAG experiment with non-pH neutralised NAC, each treatment arm was performed in triplicate. The results shown in Table 6.4 for each of the experiment arms was the mean of three values. Excel was used to record the data. A paired t-test was used to compare the mean drop in optical density of ECS following addition of NAC. On the second run of the GAG experiment comparing neutral NAC with NAC of pH 1.4, for each test arm two samples were prepared, one was processed using the GAG assay and one for the cellulose acetate electrophoresis. This resulted in a single sample for the GAG assay. On retrospective reflection, the author felt triplicate samples should have been used to allow similar statistical analysis of the data as in the first run of the experiment.

6.7 Cellulose acetate electrophoresis

Figure 6.2 Picture of cellulose acetate electrophoresis assay used to investigate the effect of NAC on ECS.

Cellulose acetate horizontal electrophoresis unit was purchased from Scie Plas (Cambridge, UK) (Figure 6.2). Pyridine - based buffer was made up in the following ratios: (pyridine : acetic acid : deionised H_2O) (10 : 1 : 89 v/v). 200mL of buffer was added to each of the two compartments. Cellulose acetate paper was labelled with the different samples using a pencil. The strips were pre-soaked in buffer for 5 minutes. Filter paper was used to form a bridge between the buffer from one of the compartments and the cellulose acetate (CA) strip on the other side. There needed to be two filter papers, one for each compartment with the CA strip sandwiched between the two filter papers. The CA strip needed to be in direct contact with the filter papers with an overlap area of at least 0.5cm to ensure transfer of current through the CA strip. After applying the CA strip, it was left to dry from previous soaking in buffer and left to run for 10 minutes at a voltage of 70V prior to adding the individual samples onto the strip. 5μL of each sample was applied to the CA strip. The strip was left to run on a voltage of 70V for 1 hour then removed and placed in an aqueous alcian blue stain for 15 minutes (STAIN). Then it was placed into sodium chloride - saturated absolute ethanol (DESTAIN). The strip was removed from the DESTAIN step and placed between two blotting papers after which it was left overnight to dry.

6.8 Effects of NAC on ECS

The biofilm - reducing characteristic of NAC may be secondary to pH, or to the direct action of NAC molecule on the ECS of biofilm. To investigate this, two sets of experiments were performed. Initially, non-pH adjusted NAC was used, then non-pH adjusted NAC and neutralised NAC were compared. For the first experiment the controls were chondroitin sulphate (CS), de-ionised water (dH_2O), and ECS. Non-pH adjusted NAC was then added to ECS both with and without sodium azide (NaN_3). NaN_3 was added to see if during the incubation period the samples had bacteria that were replicating thus giving an erroneously high reading, introducing error into the GAG measurement. Each arm was performed in triplicate and the average of the results was calculated. All samples were incubated for 3.5 days in 37°C to allow reaction of the different mixtures to occur. Following the incubation period 1 mL of each of the solutions was run through the alcian blue GAG assay.

The seven arms of the experiment were as follows: (Non pH-adjusted NAC)

dH_2O control: 1mL of de-ionised water
CS control: 1mL of 25mg/mL chondroitin sulphate
ECS control: 1mL of ECS solution
ECS + NAC: 1mL of ECS solution + 100μL of 50mg/mL of NAC
ECS + NaN_3: 1mL of ECS solution + 100μL of 10mgs/mL of NaN_3
ECS + NaN_3 + NAC: 1mL of ECS solution + 100μL of 10mgs/mL of NaN_3 + 100μL of NAC

The results for non-pH adjusted NAC were as follows (Table 6.4):

Controls	**Reading (A490nm OD)**	**Experiment arm**	**Reading (A490nm OD)**
dH_2O	0.003	ECS + NAC	0.236
CS	0.082	ECS + NaN_3	0.493
ECS	0.532	ECS + NaN_3 + NAC	0.283

Table 6.4 Results of the alcian blue GAG assay of the different experiment arms after 3.5 days of incubation at 37°C. dH_2O: de-ionised water, CS: chondroitin sulphate, ECS: extra-cellular slime, NAC: Non pH-adjusted N-acetylcysteine, NaN_3: sodium azide. The table shows a marked reduction in the alcian blue reading when NAC was added to ECS (p=0.003). NaN_3 did not alter the readings and therefore the effect observed was not due to bacterial presence.

The negative control, de-ionised H_2O, gave very low reading meaning there was minimal background interference from contamination. As expected the positive control, CS, gave a reading of 0.082 similar to previous results quoted for this assay (Bayston & Rogers, 1990). Extra-cellular slime gave the highest reading of 0.532. It would be expected that if there were bacteria replicating in the samples during the incubation period, then the ECS sample without the toxic agent NaN_3 would have a much higher reading than ECS with NaN_3, however this was not the case as both gave similar readings; ECS = 0.532, ECS + NaN_3 = 0.493.

The addition of non pH-adjusted NAC to ECS resulted in almost a half reduction in the amount of GAG's. ECS = 0.532, ECS + NAC = 0.236 (p=0.003). This was again similar when NaN_3 was present to avoid bacterial contamination and interference with the results; ECS + NaN_3 = 0.493, ECS + NaN_3 + NAC = 0.283 (p=0.005).

This implies one of two things: either half the ECS precipitates after addition of NAC, or, that NAC breaks down the ECS into half its quantity. There was a considerable amount of ECS that precipitated after the addition of NAC, and this precipitate may have been removed in the alcian blue assay during the ethanol-washing step. As a separate experiment, no precipitation was observed on adding NAC to alcian blue alone. For this reason it is more probable that the reduction in measured ECS quantity following addition of NAC was due to precipitation.

On the previous run, following the addition of NAC to ECS, this was then incubated for 3.5 days at 37°C prior to analysis of the samples. Due to the observation that if precipitation were to occur it happened immediately, this incubation period was not deemed necessary and therefore the samples were processed directly on the repeat run. Also NaN_3 was not added to the mixtures because as previously explained there was no bacterial contamination that could have affected the readings. On the repeat run, both neutralised and non-pH - adjusted NAC were compared to examine the effects of pH.

There were seven arms as follows:

dH_2O control: 1mL of de-ionised water
Neutralised NAC: 1mL of dH_2O + 100µL of 50mg/mL neutralised-NAC
Non-pH - adjusted NAC (pH 1.4): 1mL of dH_2O + 100µL of 50mg/mL non-pH adjusted NAC
CS control: 1mL of 25mg/mL chondroitin sulphate
ECS control: 1mL of ECS solution
ECS + Neutralised NAC: 1mL of ECS solution + 100µL of 50mg/mL of neutralised-NAC
ECS + non-pH - adjusted NAC: 1mL of ECS solution + 100µL of 50mg/mL of non-pH adjusted NAC

Alcian blue GAG assay:

Controls	**Reading (A490nm OD)**	**Arms**	**Reading (A490nm OD)**
dH_2O	0.000	ECS + NAC (neutral)	0.535
NAC (neutral)	0.009	ECS + NAC (pH 1.4)	0.306
NAC (pH 1.4)	-0.001		
CS	0.116		
ECS	0.552		

Table 6.5 Results of the alcian blue GAG assay of the different experiment arms. dH_2O: de-ionised water, CS: chondroitin sulphate, ECS: extra-cellular slime, NAC: N-acetylcysteine. Minimal reduction in ECS quantity was observed on addition of neutral NAC to ECS, in contrast addition of NAC (pH 1.4) to ECS resulted in a considerable reduction in measured ECS.

From the results (Table 6.5), it appears that the reduction in ECS quantity was pH - related, as there was minimal difference in the reading between ECS and ECS + NAC (neutral). This was in contrast to a large reduction in ECS when using NAC (pH 1.4).

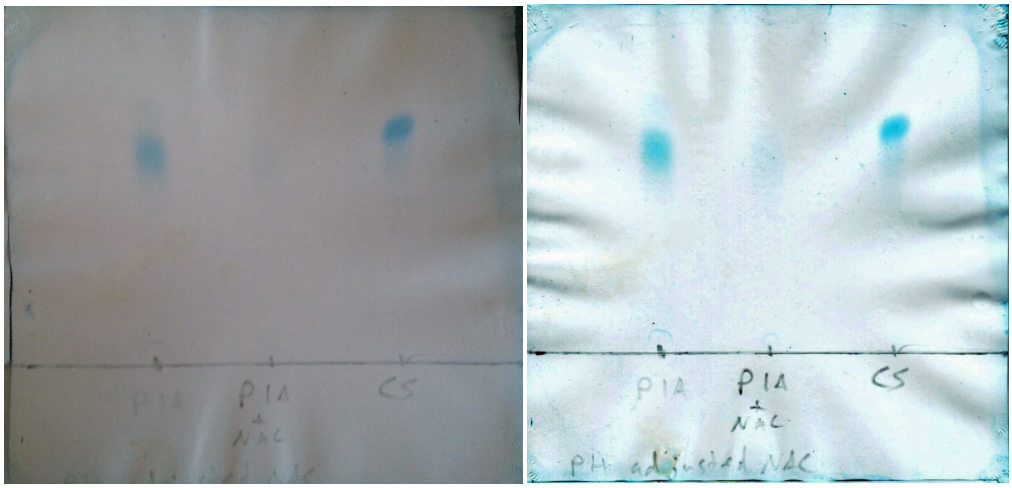
Cellulose acetate electrophoresis:

In order to obtain further information about the effect of NAC on the molecule size of ECS the cellulose acetate electrophoresis assay was performed.

The first run of electrophoresis had the following arms:

CS control: 1mL of 25mg/mL chondroitin sulphate
PIA control: 1mL of ECS solution
PIA + NAC: 1mL of ECS solution + 100µL of 50mg/mL of neutralised-NAC

When applying a sample of each of the above arms onto the cellulase acetate paper, a 10µL pipette was used and 1µL of each sample was applied onto a line drawn in pencil (Figure 6.3)



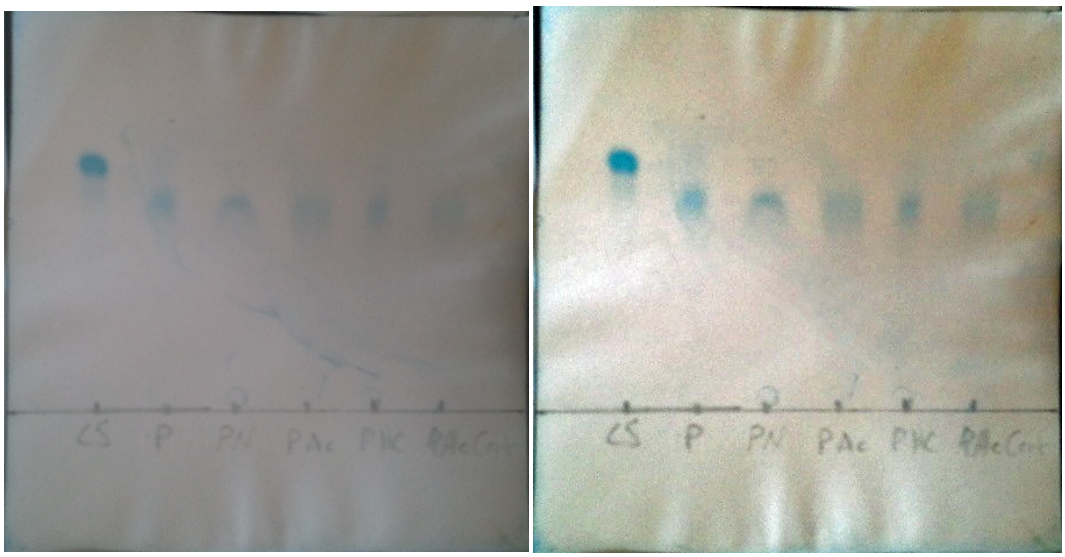
Original photo Rendered photo

Figure 6.3 CA electrophoresis showing the effects of adding neutralised NAC to polysaccharide intercellular adhesin (PIA), representing ECS. CS: chondroitin sulphate control

The results of the first run of CA electrophoresis showed there was a considerable reduction in the staining of PIA (the main ECS of *S aureus*) following addition of neutralised NAC (Figure 6.3) when compared to PIA alone and the CS control. This reduction may indicate a breakdown of PIA. The majority of the stain in the PIA and the PIA + NAC had travelled a similar distance suggesting that the remaining PIA in the NAC arm was of same molecular size to the original PIA. Also there was a halo seen above the PIA stain that was not present following addition of neutralised NAC. This halo may represent a smaller sized molecule that had travelled faster than the rest of PIA. The absence of this halo following NAC addition may indicate a chemical reaction where this molecule changed structure so that it was no longer detectable on the CA paper.

The second run of CA electrophoresis explored whether the effects of non-pH - adjusted NAC on ECS was purely pH - related, or whether NAC at a low pH had an effect on ECS that cannot be replicated by the addition of acid on its own. For this reason the following arms were prepared:

CS control: 1mL of 25mg/mL chondroitin sulphate
P: 1mL of ECS solution (PIA)
PN: 1mL of ECS solution + 100µL of 50mg/mL of non-pH adjusted NAC
PAc: 1mL of ECS solution + acetic acid titrated till turbidity appeared
PHc: 1mL of ECS solution + hydrochloric acid titrated till turbidity appeared
PAcCent: 1 mL of ECS solution + acetic acid titrated till turbidity appeared, then sample centrifuged at 3000rpm and supernatant tested



Original photo Rendered photo

Figure 6.4 CA electrophoresis showing the effects of adding non-pH adjusted NAC to polysaccharide intercellular adhesin (PIA), in comparison with hydrochloric acid and acetic acid. CS: chondroitin sulphate control, P: PIA, PN: PIA + non-pH adjusted NAC, PAc: PIA + acetic acid, PHc: PIA + HCl, PAcCent: supernatant following centrifugation of PAc. The figure shows a reduction in staining of PIA + NAC and disappearance of a halo when compared to PIA alone, this was not replicable by the addition of hydrochloric acid or acetic acid.

The results showed that similar to the previous run of CA electrophoresis, there was a reduction in the staining of PIA + NAC as compared to PIA alone. Also the halo in front of the PIA control was not apparent following addition of NAC. This halo was still present on addition of hydrochloric acid or acetic acid. This was not easy to appreciate therefore the CA paper was illuminated with a light from behind and a picture taken to illustrate the points mentioned (Figure 6.5).

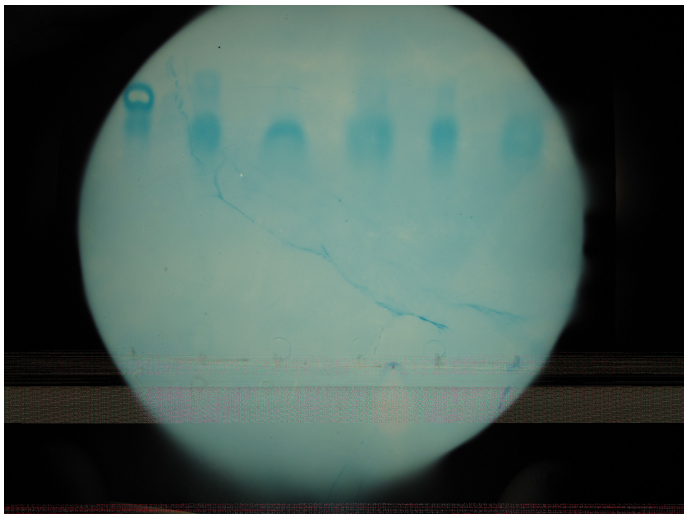


Figure 6.5 CA paper with light shone from behind to illustrate a halo in front of PIA stain (second stain from the left), not present in the PIA + NAC stain (third stain from left). Halo was still present with PIA + acetic acid (fourth stain from left) and PIA + HCl (fifth stain from left). The figure indicates that the effect of adding NAC on PIA was not purely pH – related, because similar effects were not replicable on adding hydrochloric acid or acetic acid.

These results indicate that the effects of NAC were not purely pH - related. The significance of the halo that disappears on addition of NAC is not known, however we postulate that this may represent a smaller molecule of the PIA that was degraded by NAC. Finally it was important to perform a direct comparison between neutralised NAC and non-pH adjusted NAC. The following arms were prepared:

CS control: 1mL of 25mg/mL chondroitin sulphate
PIA: 1mL of ECS solution (PIA)
PIA + NAC pH 1.4: 1mL of ECS solution + 100µL of 50mg/mL of NAC at pH 1.4
PIA + NAC pH 7.3: 1mL of ECS solution + 100µL of 50mg/mL of NAC at pH 7.3

Original photo Rendered photo

Figure 6.6 CA electrophoresis comparing the addition of neutralised NAC and non-pH adjusted NAC to PIA, the main ECS of *S aureus*. CS: chondroitin sulphate, PIA + NAC pH 1.4 (second stain from right), PIA + NAC pH 7.3 (first stain right side).

The results of this run illustrate that the PIA stain was reduced similarly following the addition of either neutralised or non-pH - neutralised NAC (Figure 6.6). To illustrate this further a light was illuminated behind the CA paper (Figure 6.7). Due to artefact from the staining procedure the halo in front of the PIA was not as apparent in this run.

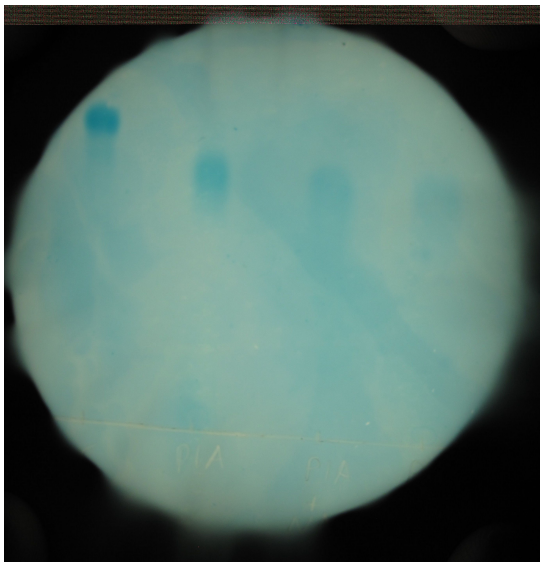


Figure 6.7 CA electrophoresis showing the reduction in PIA stain, the main ECS of *S aureus*, following the addition of either neutralised or non-pH neutralised NAC. Chondroitin sulphate (1st stain on left), PIA (2nd stain from left), PIA + NAC pH 1.4 (third stain from left), PIA + NAC pH 7.3 (first stain on right). Reduction in stain may represent degradation of PIA molecule, a major biofilm constituent, by NAC. This was replicable on neutralising NAC.

The result from the CA electrophoresis has added important clues to the mechanism of action of NAC. The importance of pH was not as apparent and in fact all the changes to the ECS stain following addition of NAC were comparable whether pH neutralised or not. These changes were not replicable by addition of an acid suggesting this was the direct effect of NAC. The findings suggest that PIA has two components: a large molecule making up the bulk of PIA, and a smaller molecule that is present in less quantity seen as a halo on the CA paper. The addition of NAC regardless of pH caused the disappearance of the halo, thus indicating that the smaller molecule was no longer detectable. These findings were not apparent on the alcian blue GAG assay that gave different kind of information: the addition of neutralised NAC did not affect the total measurable ECS, but a low pH regardless of NAC caused precipitation that affected the final reading. This indicated that ECS precipitated but did not change molecular structure on lowering pH because the turbidity caused in the ECS was reversible on reversing pH. To fully appreciate the effects of NAC on ECS both assays need to be interpreted together.

6.9 Discussion

Based on the observations and results of the alcian blue GAG assay, low pH causes insolubility of ECS. This might cause the disruption of biofilm structure, equally it might make biofilms less permeable. To see how this translates to live biofilms a separate assay would need to be performed to test the effect of low pH on biofilms without the use of NAC. The CA electrophoresis assay demonstrated that low pH did not affect the molecular structure of ECS, but NAC regardless of pH appeared to have an effect on ECS. This effect was apparent by a reduction in the PIA stain on the CA paper and disappearance of a halo possibly representing a smaller molecule. Both *S epidermidis* and *S aureus* carry the *icaADBC* gene locus that is responsible for PIA production (McKenney *et al*., 1999). PIA of *S epidermidis* was demonstrated to consist of two types of polysaccharides that are similar in structure (Mack *et al*., 1996). Major polysaccharide I was heavily N-acetylated and minor polysaccharide II was moderately anionic, they were present at a ratio of 7:1 respectively (Mack *et al*., 1996). It is possible that in this study the darker stain of PIA on the CA electrophoresis paper represented the major N-acetylated polysaccharide I, and the smaller halo represented the minor neutral polysaccharide II thus reflecting a reduction in polysaccharide II by NAC. It was not possible to make confident assertions about this because it was not known if the PIA produced by *S aureus* - F2315 had similar polysaccharide composition to that of *S epidermidis*. As PIA is composed of repeat units of N-acetyl-glucosamine, a monosaccharide derivative of glucose, with a β-1,6 formation, a potential mechanism of degradation by NAC may involve breaking the linkages between the single units of N-acetyl-glucosamine causing the reduction in the PIA stain. These results provide important clues about the mechanism of action of NAC however further experiments are needed to verify the exact biochemical pathways involved.

More importantly it was important to see whether the effects of neutralised NAC on ECS translated into a similar effect on live biofilm. From the results of Section 5 one cannot be sure if biofilm eradication was due to pH or NAC. Therefore the effects of neutralised NAC and low pH NAC needed to be tested against live biofilms both with and without antibiotics. With multiple combinations, the silicone disc biofilm assay would be too labour - intensive to perform. Also the assay was not suited to examine multiple concentrations of antibiotics against multiple concentrations of NAC in order to find the optimum mixture. Therefore we employed a high throughput technique called the MBEC assay described in further detail in the next section.

Section 7. Minimum Biofilm Eradication Concentration (MBEC) Assay

7.1 Introduction

Antibiotic susceptibility is generally measured using MIC values that measure bacterial sensitivity to antibiotics in planktonic free-floating suspension form. The MIC value generally guides clinical dosage of antibiotics. The major flaw with this is the assumption that bacteria in infections are present in planktonic form. The growing evidence shows that many chronic infections are caused by bacteria present in biofilm state such as that presented here with regard to middle ear biofilms in OME. Measurement of bacterial susceptibility to antibiotics in the biofilm state requires an assay that can reliably process batch cultures of biofilms with reproducible results with the ability to test biofilms against a concentration gradient of antibiotics. This poses major practical difficulties if the silicone biofilm disc assay described in section 5 is used. A biofilm research group based in Calgary, Alberta, Canada have developed a high-throughput technique to test antimicrobial agents against mature biofilms grown on pegs that are attached to a microtitre plate with the ability to control the exposure conditions. This is called the minimum biofilm eradication concentration (MBEC^{TM}) assay that essentially describes the minimum concentration of antibiotic needed to eradicate a bacterial biofilm of a specific bacterial species (Harrison *et al*., 2010).

The MBEC^{TM} assay suited the requirements of this project though adaptations were needed to allow the testing of various rifampicin/clindamycin concentrations against various concentrations of NAC both with and without pH neutralisation. In order to do this, the basic principles of broth microdilution checkerboard testing for synergy were adopted (Moody 2004). The results would give a closer approximation to the realistic doses required for local delivery of antimicrobials to the middle ear for the eradication of biofilms when compared to MIC values. This setting would also allow

a more realistic evaluation of the synergistic effects of NAC with antibiotics against bacterial biofilms both with and without the previously observed potent action of low pH.

7.2 Methods

The overall principle behind the MBEC assay rests on growing biofilms on plastic pegs attached to the cover of a 96 well microtitre plate. Firstly the cryogenic stock of the bacteria was subcultured twice to obtain a fresh specimen. This was suspended in broth and the MIC of the inoculum as well the species was verified to there has been no bacterial resistance or contamination. The MBEC device (96 well plate with cover containing plastic pegs) was inoculated and incubated for 4 days to grow mature biofilms. A challenge plate with antimicrobials was set up in the meantime to the desired combination of antimicrobials. The biofilm pegs were added onto the challenge plates for 48 hours, 1 week, and 2 weeks. Following the exposure period, the biofilm-cultured pegs were removed from the challenge plate, and rinsed twice using 96 well plates filled with PBS. The biofilm pegs were then placed in a recovery medium containing universal neutraliser to neutralise any further effect of the antimicrobials and allow the surviving bacteria to grow. The pegs immersed in recovery medium were then sonicated to disrupt the biofilms. The challenge plates were separately processed looking at turbidity using a 96 well plate optical reader at 490nm to work out the MIC values. The recovery plates were incubated for 38 hours following which viable cell counts were performed to quantify the biofilms and work out the MBEC values.

MBEC™ assay: summary of steps

Verify inoculum

↑

Cryogenic stocks → First subculture → Second subculture → Resuspension in broth

↓

Inoculate MBEC device

↓

Incubate on gyrorotary shaker for 4 days

↓

Viable cell counts (MBEC value) — Rinse pegs

↑ ↓

Recovery medium — Set up challenge plate

↑ ↓

Disrupt biofilms ← Biofilm cultures 96 pegs ← Exposure to antimicrobials for desired period

↓

Planktonic cultures 96 wells

↓

Optical reader at 420nm

(MIC value)

Basic experiment layout (6 MBEC plates)

As the comparison needed was between pH-neutralised NAC and non-pH neutralised NAC, the same experiments were performed for each in parallel with antimicrobial exposure periods of 48 hours, 1 week, and 2 weeks. Therefore 6 MBEC plates were needed for the experiment.

pH-neutralised NAC:
Antimicrobial exposure periods: 48 hours 1 week 2 weeks

non-pH neutralised NAC:
Antimicrobial exposure periods: 48 hours 1 week 2 weeks

Preparing the MBEC plates:

Each of 96 wells of a microtitre plate were filled with 150 µL of inoculum. There were six MBEC plates, therefore the total amount needed was 96 x 150 µL x 6 = 86,400 µL = 86 mL of inoculum. The target inoculum needed, a 1 : 30 dilution of a 1.0 McFarland suspension, was achieved by preparing fresh overnight subcultures of *S aureus* F2315. Ten colonies were picked up using a sterile cotton wool stick and mixed in 10 mL of TSB giving an optical density of 0.219 at 620nm (1.0 McFarland = 0.25 OD). Then 5 mL of this suspension was added to 145 mL of TSB giving a 1 : 30 dilution required and a total volume of 150 mL (86 mL was needed). Separate samples were taken from the broth 1 : 30 dilution to verify the MIC and API staph to confirm the bacterial identity. MIC was performed on ISA plates against rifampicin and clindamycin as per protocol in section 3.2.3. Clindamycin MIC was 0.064 mg/L, and rifampicin was 0.003-0.004 mg/L, resembling previous MIC for *S aureus* F2315 and hence no resistance has developed. The API staph was also identical (6336143 92% *S aur*eus). All wells had 150 µL of inoculum apart from the sterile controls A11, A12, B11, and B12 that contained 150 µL of TSB. The configuration of the wells was as shown in Table 7.1.

To check the CFU's of inoculum, serial dilutions were performed and 200 µL of each dilution was spread on agar plates and incubated overnight. The 10^{-3} dilution of inoculum grew 732 colonies on the plate, therefore 732 x 5 = 3660 CFU / mL (5 x 200 µL = 1 mL). If 3660 CFU / mL was for the 10^{-3} dilution, then the original 1 : 30 dilution inoculum was 3.66×10^{6} CFU / mL. Each well had 150 µL, ie. Each inoculum contained 3.66×10^{6} CFU / mL x 0.150 mL = 5.49×10^{5} colonies per 150 µL well.

Rif/Clind MIC														
A	10,000											Sterile	Sterile	
B	10,000											Sterile	Sterile	
C	1000													
D	1000													
E	100													
F	100													
G	0	Growth	Growth											
H	0	Growth	Growth											
		0	0	0.005	0.005	0.05	0.05	0.5	0.5	5	5	50	50	NAC (mg/mL)
		1	2	3	4	5	6	7	8	9	10	11	12	Well columns

Table 7.1 MBEC 96 well microtitre plate layout. The four growth control wells had no antimicrobial applied. The four sterile controls had no inoculum or antimicrobial applied. The rest of the wells were arranged so that NAC was applied in increasing concentration gradient against combination of rifampicin/clindamycin in a similarly increasing concentration gradient. The peg containing lids with biofilm grown on them were placed into the 96 wells. The four pegs going into the sterile control wells had no biofilm to test for sterility of the assay.

Following preparation of the 96 micro-titre wells (Table 7.1), the MBEC peg lids were placed onto each of the plates so that the plastic pegs immersed into the wells. For the biofilms to grow on the pegs, the plates were incubated in 37°C using an orbital shaker at 120 rpm and left for 4 days to develop mature biofilms. After the incubation period, each of the 6 biofilm-cultured peg lids were rinsed to remove loosely adherent planktonic bacteria. This was performed by preparing a 96 well plate with 200 µL of PBS in each of the wells. The biofilm-cultured peg lids were each immersed into a PBS rinse plate and left for 2 minutes. The biofilm-cultured peg lids were transferred to previously prepared antimicrobial challenge plates as described below.

Antimicrobial challenge plates:

Using the 96 well plate configuration shown in Table 7.1 each of the possible combinations was performed in quadruplicate. There were four sterile controls, four growth controls, and four wells of any given combination of NAC and rifampicin/clindamycin.

The concentrations for the antibiotics used were 10000 MIC, 1000 MIC, and 100 MIC. The concentrations of the NAC used were 50, 5, 0.5, 0.05, and 0.005 mg / mL. This configuration was to facilitate the serial 1 in 10 dilution method for preparing the micro-titre wells. There were 24 wells for each of the MIC concentrations, and 6 challenge plates. Each of the wells was filled with a total of 200 µL of the challenge solution, to ensure complete submersion of the biofilm in the antimicrobial. This consisted of 100 µL of the antibiotic solution and 100 µL of the NAC solution (either pH neutralised or non-pH neutralised), each at double strength to achieve the required target concentration. The total amount of antibiotic solution needed for each given MIC was 24 x 6 x 100 µL = 14400 µL = 14.4 mL at double strength, 20000 MIC. The total amount of NAC needed was 16 wells for each of the given NAC concentrations between 6 plates, ie. 16 x 6 x 100 µL = 9600 µL = 9.6 mL of 100 mg / mL NAC.

20000 MIC of rifampicin / clindamycin solution
= Rif 60 µg / mL + Clind 2000 µg / mL

10000 MIC of rifampicin / clindamycin solution
= Rif 30 µg / mL + Clind 1000 µg / mL

A 50 mL quantity of 20000 MIC Rif/Clind stock solution was made by adding 25 mL of 40,000 MIC rifampicin (120 µg / mL) and 25 mL of 40000 MIC of clindamycin (4 mg / mL).

The four sterile control wells and the four growth control wells on the challenge plate had 200 µL of TSB each. The pegs that were inserted into the sterile controls should not have grown any biofilm as they were immersed in sterile TSB. The pegs that were inserted into the four growth control wells should have biofilm growth on them, and should have shown most growth because there was no antimicrobial challenge to the biofilms.

Following the 'challenge' period the biofilm-cultured peg lids were removed from the challenge plates and rinsed twice in PBS using sterile microtitre plates filled with 200µL of PBS in each of the wells. They were then transferred to the recovery plates to neutralise any left over antimicrobial and allow surviving bacteria to grow. The 96 well plates containing the antimicrobial challenge were examined for turbidity to determine MIC values by placing the plates through an optical plate reader (Biotek EL800, KC4 v3.4 software) and read at 490nm.

Preparing recovery plates:

A surfactant - supplemented growth medium was prepared with universal neutraliser solution added to make up the recovery medium. The desired constituents for the growth medium were 1 litre of TSB, 20 gm / L saponin, and 10 gm / L Tween 80. Universal neutraliser was prepared using 1 gm L-histidine, and 1 gm L-cysteine, dissolved in 20 mL of distilled water then filter sterilised using 0.20 µm filter (minisart highflow). Then, 500 µL of universal neutraliser were added to separate 19.5 mL vials of surfactant supplemented TSB for the recovery plates.

Disrupting biofilms:

Prior to disrupting the biofilms, the MBEC lids that were to be used for SEM imaging were removed and saved separately. Following this, each of the rinsed biofilm-cultured peg lids was immersed into 96 well plates filled with 200 µL of recovery medium in each well. They were then strapped with waterproof tape around the gap between the peg - containing lids and the well plates to prevent contamination during sonication. The MBEC plates were sonicated in water filled bath for 15 minutes on high (Figure 7.1). The 96 well plates containing sonicate were covered with sterile 96 well plate lids and incubated for 38 hours at 37°C.

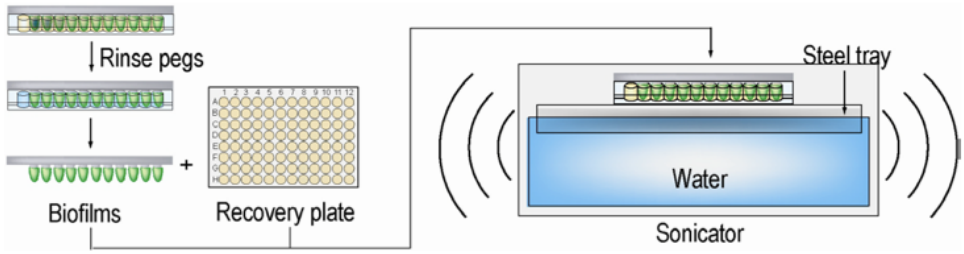


Figure 7.1 Sonication. The biofilms were rinsed twice with physiological saline and immersed into a recovery plate. This was then sonicated to disrupt the biofilms into the universal neutraliser solution.

Viable cell counts:

The viable cell count of each well of the recovery medium was measured by the serial dilution method followed by spot plating onto agar plates. A 100µL volume of each row of the recovered plate eg. A1 to A12 was transferred to the top part of a sterile 96 well plate. All the other wells of the sterile 96 well-plate (B2 to H12) were filled with 180 µL of PBS. Then 20 µL was serially diluted from A1 – A12 to H1 – H12 using a multichannel micropipette to perform 1 in 10 dilutions. Therefore each recovered plate containing 8 rows from A to H needed 8 separate sterile 96 well plates to perform the 1 in 10 dilutions. Sheep blood agar plates were then divided into 8 compartments using a black marker pen, and the serial dilutions of each column representing one well of the original recovered plate were transferred by spot plating to the agar plate marked 0 (no dilution) to -7 (10^{-7} dilution). The spot plated

agar plates were incubated at 37°C for 48 hours and viable cell counts were determined by counting the CFU's. The results were inserted into a 96 well plate format grid on excel to determine the MBEC values and examined for synergy.

Fixation of pegs for SEM:

To determine whether there was biofilm growth using SEM imaging, two of the growth control pegs from 1 week incubation period were broken from the rest of the lid using sterile forceps before sonication. Two sterile control pegs were also examined. The pegs were rinsed in 1 mL of PBS twice and fixed with 2.5% glutaraldehyde (25% glutaraldehyde diluted 1 in 10 with sterile water). This was incubated for 4.5 hours at room temperature - 20°C. The pegs were removed from the glutaraldehyde and rinsed with water. Then 3 drops of tetramethylsilane was added and left overnight to dry.

The pegs were then gold - coated for 300 seconds using a Leica sputter coater (LEICA EM SCD005). The pegs were mounted onto 0.5" aluminium specimen stubs with the help of 12mm carbon tabs and examined under SEM using JEOL SEM Control User Interface Version 6.57 software.

7.2.1 Data analysis

The challenge plate was prepared so that each test arm was performed in quadruplicate. To calculate the MIC values following 48 hours of incubation, the mean of four wells for each test arm was shown in Tables 7.2 & 7.3 in the results section. When calculating the viable cell counts following 48 hours, 1 week, and 2 week exposure, two pegs for each treatment arm was processed in duplicate and the mean of each duplicate sample was shown in Tables 7.4 to 7.9 in the results section. As the final outcome was eradication of biofilm with a viable cell count of zero, statistical analysis was not necessary. Excel spreadsheet was used for collecting the data and processing the means.

7.3 Results

The results are depicted so that only the averages of the quadruplicates are presented.

MIC results:

The antimicrobial challenge plates following 48 hours of incubation with the biofilm-cultured peg lids were examined under the optical plate reader to give the following results;

Non-pH neutralised NAC (OD 490nm)

Rif/Clind MIC	0	0.005	0.05	0.5	5	50	NAC mg/mL
10000	0.298	0.24825	0.27275	0.314	0.19675	0.12475	
1000	0.15575	0.152	0.16	0.1635	0.10225	0.09325	
100	0.1405	0.14275	0.14425	0.14775	0.09125	0.08575	
0	0.68575	0.6275	0.57425	0.58525	0.507	0.08675	

Table 7.2 Optical density readings (OD 490nm) of the antimicrobial challenge plate testing non-pH neutralised NAC against antibiotic combination rifampicin / clindamycin after 48 hours incubation with biofilm-cultured MBEC peg lids. Each number was the average of four wells tested in quadruplicate. Dark shading: growth control 0.68575, sterile control 0.12475. All wells were clear meaning inhibition apart from the growth control and NAC concentrations 0.005 to 5 mg / mL without antibiotics in medium shade. Therefore the MIC of non-pH - neutralised NAC was between 5 and 50 mg / mL.

Analysing the MIC values from the OD readings (Table 7.2), all wells were clear meaning inhibition apart from the growth control and NAC concentrations 0.005 to 5 mg / mL without antibiotics in medium shade. Therefore the MIC of non-pH - neutralised NAC was between 5 and 50 mg / mL. This confirms previous results from section 6.4 showing MIC of non-pH neutralised NAC to be 9mg / mL. The inhibiting point of antibiotic combination rifampicin/clindamycin is between 0 and a 100 MIC. The reason that the readings were slightly higher at 10000 MIC is that the increased concentration of rifampicin gave a red colour (between 0.19675 and 0.314). Visual assessment of those wells showed no turbidity but dark red colour.

pH-neutralised NAC (OD 490nm)

Rif/Clind MIC							
10000	0.33525	0.33125	0.333	0.37725	0.3365	0.11325	
1000	0.212	0.18575	0.20775	0.2105	0.1615	0.137	
100	0.19575	0.16575	0.179	0.1765	0.151	0.143	
0	0.7035	0.583	0.5715	0.5755	0.6135	0.59025	
	0	**0.005**	**0.05**	**0.5**	**5**	**50**	**NAC mg/mL**

Table 7.3 Optical density readings (OD 490nm) of the antimicrobial challenge plate testing pH-neutralised NAC against antibiotic combination rifampicin / clindamycin after 48 hours incubation with biofilm-cultured MBEC peg lids. Each number was the average of four wells tested in quadruplicate. Dark shading: growth control 0.7035, sterile control 0.11325. All wells of the antibiotic combination rifampicin / clindamycin 100 MIC and above were clear meaning inhibition. All the wells of the NAC concentrations 0.005 to 50 mg / mL without antibiotics in medium shade were turbid with high OD readings. Therefore the MIC of pH - neutralised NAC was not reached in this assay.

The area in medium shading revealed that pH-neutralised NAC without antibiotics did not inhibit bacteria up to the maximum concentration used: 50 mg / mL (Table 7.3). This confirms results from section 6.4 showing that pH neutralisation of NAC

increases the MIC to 160mg / mL, above the concentration tested in this assay. Similar to the previous results 10000 MIC darkened the colour of the wells thus giving higher OD readings (0.33125 – 0.37725). Visual assessment of the wells revealed no turbidity but a dark red colour. Again the inhibiting point of antibiotic combination rifampicin/clindamycin was between 0 and 100 MIC.

Viable cell counts:

With the viable cell count technique, results were performed in duplicate using the average from the counts of two wells.

Exposure for 48 hours:

Non-pH neutralised NAC

Rif/Clind MIC							
10000	850	1750	350	150	0	0 - sterile	
1000	2.85×10^9	1.75×10^9	3.28×10^9	2×10^9	2.15×10^9	0	
100	2.25×10^9	2.1×10^9	2.65×10^9	2×10^9	4.25×10^9	0	
0	2.50×10^9	3.45×10^9	1.9×10^9	2.5×10^9	2.1×10^9	0	
	0	**0.005**	**0.05**	**0.5**	**5**	**50**	**NAC mg/mL**

Table 7.4 Results of CFU readings from the MBEC peg lids following biofilm quantification by the viable cell count method after 48 hours exposure to non-pH neutralised NAC at various concentrations with combination of rifampicin / clindamycin. The numbers were the average of two wells tested in duplicate. Dark shadowing: growth control 2.5 x 10^9 CFU's, Sterile control: 0 CFU's. The results showed that biofilms were eradicated with 50 mg / mL of NAC on its own and also with 5 mg / mL of NAC in combination with 10000 MIC of antibiotics.

After 48 hours exposure, biofilms were eradicated with non-pH - neutralised NAC at 50 mg / mL both with or without antibiotics, and also at 5 mg / mL NAC with 10000 MIC rifampicin / clindamycin (Table 7.4). There was considerable inhibition of biofilm using 10000 MIC compared to 1000 MIC of antibiotics without NAC (850 versus 2.85 x 10^9). These results resemble similar findings of the silicone disc biofilm assay, where non-pH - neutralised NAC at 50 mg / mL without antibiotics inhibited 5 day - old biofilms after 24 hours of exposure and eradicated biofilm after

7 days (see section 5.3). More importantly the effects of NAC needed to be examined when the pH has been neutralised.

pH neutralised NAC

Rif/Clind MIC	0	0.005	0.05	0.5	5	50 mg/mL NAC
10000	2.5×10^6	5250	1500	275	25	0 - sterile
1000	2.75×10^9	2×10^9	2.5×10^9	1.18×10^9	1.75×10^9	5.75×10^8
100	3×10^9	1.85×10^9	2.25×10^9	2.5×10^9	1.43×10^9	1.55×10^9
0	4.5×10^9	6.83×10^8	1.55×10^9	2.5×10^9	4×10^9	3.25×10^9

Table 7.5 Results of CFU readings from the MBEC peg lids following biofilm quantification by the viable cell count method after 48 hours exposure to pH-neutralised NAC at various concentrations with combination of rifampicin / clindamycin. The numbers were the average of two wells tested in duplicate. Dark shadowing: growth control 4.5 x 10^9 CFU's, Sterile control: 0 CFU's. None of the combinations resulted in biofilm eradication. There was reduction in biofilm quantity that was directly proportional to incremental increases in NAC concentration when used in combination with 10000 MIC of antibiotics.

It was shown that pH neutralised NAC does have a biofilm - reducing potential when used in combination with antibiotics (Table 7.5). The incremental 10 fold increase in NAC concentration dramatically reduced the biofilm quantity when used in combination with 10000 MIC of rifampicin / clindamycin (2.5 x10^6, 5250, 1500, 275, 25). For the purposes of this project, it would be very unreasonable to use 10000 MIC in the middle ear, however this has shown an important proof of concept that the effects of NAC on biofilm were not purely pH - dependent.

Exposure for 1 week:

Non-pH neutralised NAC

Rif/Clind MIC	0	0.005	0.05	0.5	5	50 mg/mL
10000	0	55000	7.51×10^7	0	0	0 - sterile
1000	1.23×10^9	3330	3.07×10^5	0	0	0
100	1.75×10^6	3×10^7	4.06×10^6	0	3×10^8	0
0	2×10^9	5.25×10^8	6.5×10^8	9.75×10^8	7×10^8	0

Table 7.6 Results of CFU readings from the MBEC peg lids following biofilm quantification by the viable cell count method after 1 week exposure to non-pH neutralised NAC at various concentrations with combination of rifampicin / clindamycin. The numbers were the average of two wells tested in duplicate. Dark shadowing: growth control 2 x 10^9 CFU's, Sterile control: 0 CFU's. There was biofilm eradication of the MBEC pegs at a 0.5 mg / mL of NAC with 100 MIC antibiotics but not at 5 mg / mL with 100 MIC. Similarly, biofilm eradication occurred at 10000 MIC of antibiotics alone but not when combined with NAC of 0.005 and 0.05 mg / mL. Bacterial identity testing confirmed there were no contaminants to account for this unexpected finding.

After 1 week exposure 0.5 mg / mL of non-pH - neutralised NAC with 100 MIC of rifampicin / clindamycin eradicated biofilm (Table 7.6). This may not make sense as the combination of 100 MIC rifampicin / clindamycin and 5 mg / mL NAC should have also eradicated biofilm because this is a higher concentration of NAC. Results were taken as an average of two well readings and when the wells were examined, one of the wells showed no growth as expected, and the other well had a reading of 6 x 10^8 CFU's. To confirm that this was not contamination, the identity of the bacteria was tested and confirmed to be F2315 with identical API reading to the original inoculum. Similarly if antibiotics alone at 10000 MIC eradicated biofilms, one

would expect that the addition of 0.005 and 0.05 of NAC should also show eradication, but this was not the case. Again the bacteria of these wells were tested and there was no contamination.

pH - neutralised NAC

Rif/Clind MIC						
10000	2.4×10^7	1.5×10^7	1.26×10^8	1.5×10^5	0	0 - sterile
1000	1×10^8	9.75×10^7	1.85×10^8	7.63×10^7	0	0
100	3.53×10^8	1.85×10^8	4.5×10^8	8.51×10^7	0	0
0	6.95×10^9	1.3×10^9	3.95×10^8	9×10^8	1.23×10^9	1.25×10^9
	0	**0.005**	**0.05**	**0.5**	**5**	**50**

NAC mg/mL

Table 7.7 Results of CFU readings from the MBEC peg lids following biofilm quantification by the viable cell count method after 1 week exposure to pH-neutralised NAC at various concentrations with combination of rifampicin / clindamycin. The numbers were the average of two wells tested in duplicate. Dark shadowing: growth control 6.95 x 10^9 CFU's, Sterile control: 0 CFU's. The optimal combination that resulted in biofilm eradication was 5 mg / mL of NAC with 100 MIC of antibiotics.

After 1 week exposure, biofilms were eradicated using the lowest concentration of antibiotics 100 MIC in combination with 5 mg / mL of pH - neutralised NAC (Table 7.7). This was not possible with antibiotics alone even at 10000 MIC.

Exposure for 2 weeks:

Non-pH - neutralised NAC

Rif/Clind MIC	0	0.005	0.05	0.5	5	50	NAC mg/mL
10000	0	0	0	0	0	0 - sterile	
1000	0	0	0	0	0	0	
100	0	0	300	0	0	0	
0	1.8×10^9	7.25×10^8	7.25×10^8	8.75×10^8	1.03×10^9	0	

Table 7.8 Results of CFU readings from the MBEC peg lids following biofilm quantification by the viable cell count method after 1 week exposure to non-pH neutralised NAC at various concentrations with combination of rifampicin / clindamycin. The numbers were the average of two wells tested in duplicate. Dark shadowing: growth control 1.8×10^9 CFU's, Sterile control: 0 CFU's. Most of the biofilms were eradicated after 2 weeks exposure in contrast to biofilms exposed to antibiofilm agents for 48 hours and 1 week. This indicated that the period of exposure was an important factor in biofilm eradication.

The toxicity of non-pH - neutralised NAC in the middle ear for 2 weeks would be the greatest concern, however for comparative purposes the results are shown above. Most biofilms were eradicated after 2 weeks of 100 MIC of antibiotics even without NAC (Table 7.8). This contrasts to the results from the silicone disc biofilm assay where after 2 weeks of exposure to antibiotics rifampicin / clindamycin at 1000 MIC (ten times higher concentration), biofilms were still present. This indicates that the two assays do not compare. The various factors that may have accounted for this difference are outlined in the discussion Section 7.4.

pH - neutralised NAC

Rif/Clind MIC							
10000	0	0	0	0	0	0 - sterile	
1000	0	0	0	0	0	0	
100	5750	0	0	0	0	0	
0	1.4×10^9	1.73×10^9	1.8×10^9	2×10^9	2×10^9	1.48×10^9	
							NAC
	0	0.005	0.05	0.5	5	50	mg/ml

Table 7.9 Results of CFU readings from the MBEC peg lids following biofilm quantification by the viable cell count method after 1 week exposure to pH neutralised NAC at various concentrations with combination of rifampicin / clindamycin. The numbers were the average of two wells tested in duplicate. Dark shadowing: growth control 1.4 x 10^9 CFU's, Sterile control: 0 CFU's. Similar to non - pH neutralised NAC, most of the biofilms were eradicated after 2 weeks exposure to neutralised NAC in combination with antibiotics.

Interestingly pH - neutralised NAC did not eradicate biofilm at any concentration when used on its own. However when combined with antibiotics at 100 MIC, even at a low concentration of 0.005 mg / mL, there was eradication of biofilm after 2 weeks of exposure (Table 7.9). It may be argued that antibiotics at a 100 MIC eradicated biofilm after 2 weeks' exposure in the previous non-pH - neutralised plate so therefore it should equally have eradicated biofilms in this plate. This may point to the variability in the behaviour of biofilms or to errors in technique. The difference however was not great, between 0 and 5750 CFU's. Nevertheless, this again confirmed that pH - neutralised NAC had a biofilm - reducing ability when used with antibiotic combination rifampicin / clindamycin.

Scanning electron microscopy:

Figure 7.2 and 7.3 show the SEM images of the sterile control peg's at x300 and x500 magnification respectively. They show a smooth surface with no visual evidence of biofilm growth following 1 week of incubation in sterile TSB. Figure 7.2 shows occasional irregularity on the surface that was felt to be manufacturing imperfections. This was in contrast to the growth control peg's that were incubated in inoculated TSB wells for 1 week (Figure 7.4 to 7.9). Figure 7.4 & 7.5 were at relatively low magnification x18 and x50, respectively. Figure 7.4 shows a demarcation line depicting where the peg was immersed in inoculate with biofilm growth on one side of the line and no growth above that line.

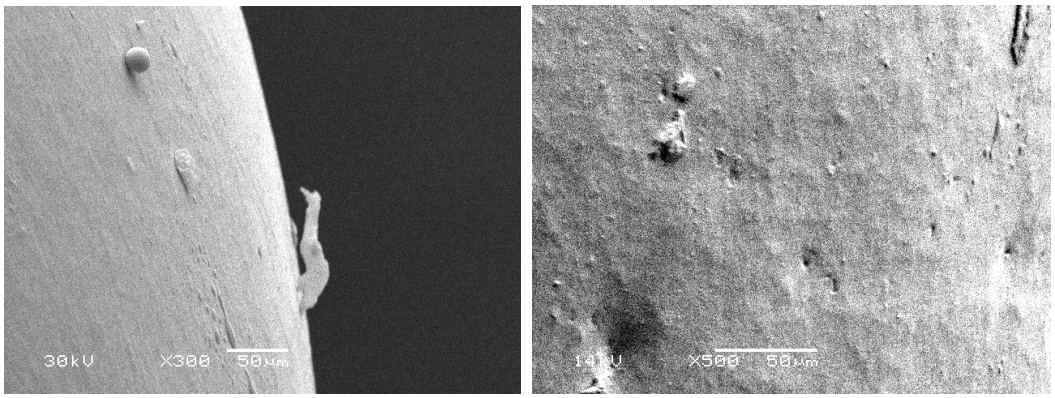


Figure 7.2 (left) & 7.3 (right) Sterile controls. Surface of sterile control plastic peg at x300 and x500 magnification shows smooth appearance with no biofilm growth.

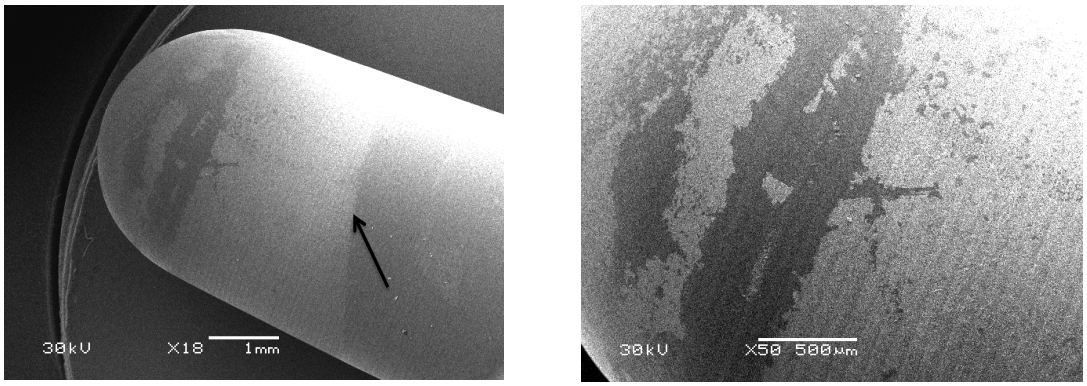


Figure 7.4 (left) & 7.5 (right) Growth control at low magnification. Growth control plastic peg's at x18 and x50 magnification. Figure 7.4 shows a demarcated line demonstrated by the arrow where the peg was immersed in inoculate and biofilm growth below the line.

Further magnification of the growth control peg's showed more detail of the biofilms. A small area deficient of biofilm (Figure 7.6 and 7.7) was purposely magnified as this helped in appreciating the difference between biofilm grown on the periphery around the central deficient area on the image. At a magnification of x2000 and x3500 (Figure 7.8 & 7.9 respectively) it was possible to visualise spherical cocci embedded in a sticky matrix with clumping of the bacteria in the biofilm.

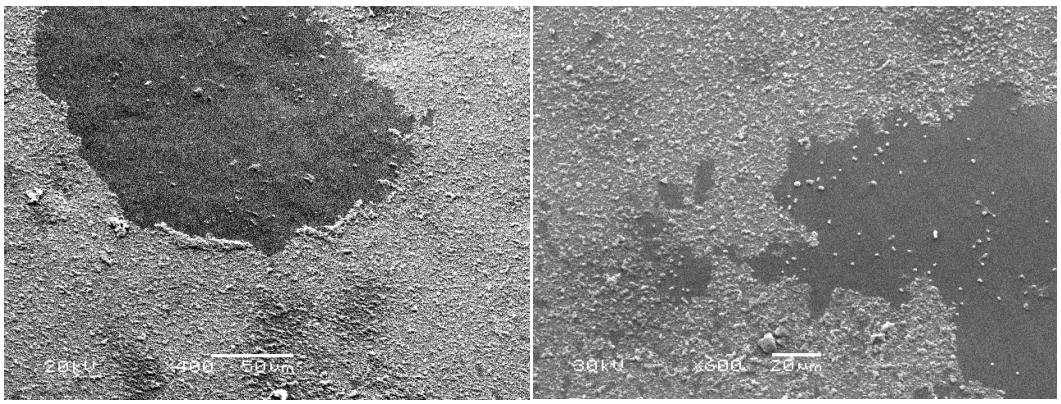


Figure 7.6 (left) & 7.7 (right) Growth control plastic peg at x400 and x600 magnification. Area deficient of biofilm in the middle of image purposely magnified to help exemplify extensive biofilm growth around this.

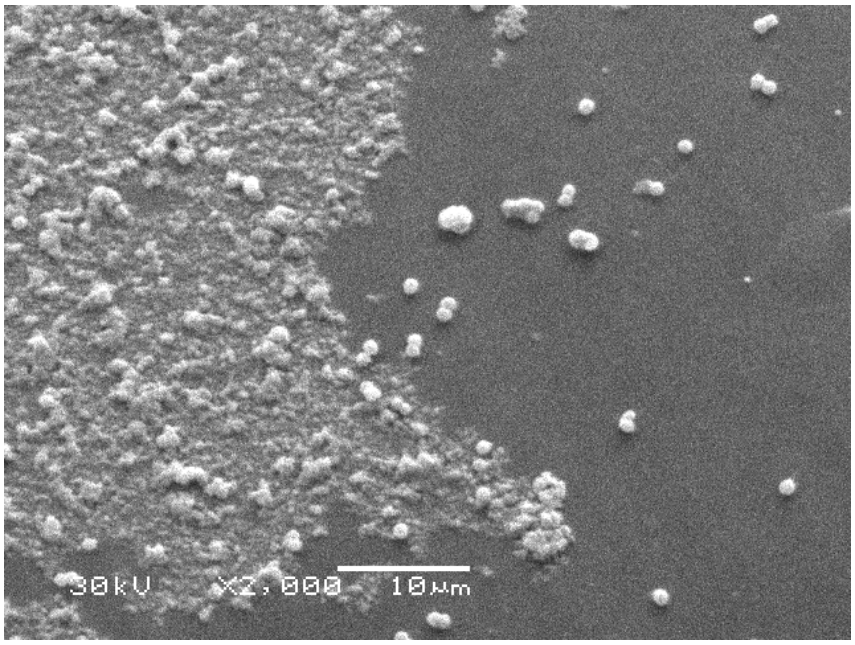


Figure 7.8 Growth control plastic peg at x2000 magnification showing spherical cocci embedded in a sticky matrix on the left of the image and without a matrix on the right of the image.

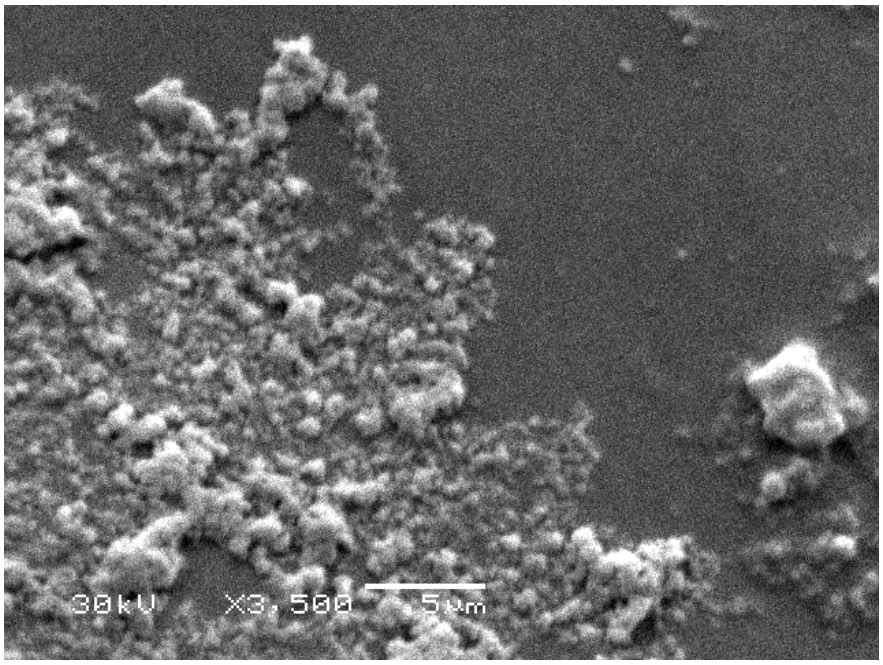


Figure 7.9 Growth control plastic peg at x3500 magnification showing clumps of bacteria embedded in sticky matrix.

7.4 Discussion

Following the results of the assays in section 5 and 6 it seemed that all the effects of NAC were purely pH - related. This posed two main questions: is it worth using NAC at all? and: can acid be used in the middle ear instead given the potency of low pH on biofilm?. Acid at high concentrations and low pH is known to be corrosive as it can react with hydrocarbons of skin or mucous membranes. Such reactions are exothermic producing heat and absorb water in the process causing destruction of cells with resultant coagulative necrosis and inflammation (Palao *et al*., 2010). This limits the use of acidic substances in the middle ear, although in vivo studies are needed to see at what concentration and pH does acid become ototoxic so that lower non-toxic concentrations may possibly be utilised. Due to this potential toxicity the need to assess the potency of pH - neutralised NAC becomes very important. The ultimate goal was to lower the antibiotic concentration needed in the middle ear to eradicate biofilms by adding a biofilm - reducing agent such as NAC. The results of this assay have conclusively shown a definite effect of pH - neutralised NAC on biofilm when used in combination with antibiotics. The assay has also demonstrated that pH - neutralised NAC on its own was not bactericidal. The greatest benefit of pH - neutralised NAC appeared to be when used in a concentration of 5 mg / mL with 100 MIC of rifampicin / clindamycin over a 1 week period. After 2 weeks this benefit was less apparent because most biofilms had had a long period of exposure to antibiotics and had therefore been eradicated regardless of NAC use.

The results of this assay showing biofilm eradication after 2 weeks' exposure to 100 MIC of rifampicin / clindamycin biofilms was in contrast to the silicone disc biofilm assay where there was clear evidence of biofilm presence after 2 weeks exposure to a higher concentration of 1000 MIC rifampicin / clindamycin. This points to differences in the assays that may have resulted in this, these are theorised as follows: firstly, 5 days – old biofilms were used in the silicone biofilm disc assay, whereas 4 days – old biofilms were used in the MBEC assay. This may have had an effect on the biofilm quantity and maturity. Secondly the method of recovery of the exposed biofilms were different: in the silicone disc biofilm assay discs that showed no growth were incubated in TSB and incubated for 5 days. In the MBEC assay all

biofilm pegs were incubated in recovery medium for a shorter duration of 36 hours prior to processing. Thirdly, the properties of the surface on which the biofilms were grown differed: the silicone discs had a rough surface whereas the MBEC pegs had a smooth surface, and it has been shown that biofilms attached on smooth surfaces are more readily disrupted than ones that attach to rough surfaces (Pasmore *et al*., 2002). It may be that biofilms attached to the smooth plastic pegs were more readily washed off in the rinse step than the biofilms attached to the rough silicone discs. It is unlikely that any one of the three factors discussed accounted for all the difference in the two assays after 2 weeks' exposure to antibiofilm and a combination of the above is the more likely explanation. Nevertheless, both assays show a proof of concept with biofilm reduction or eradication being achieved with longer exposure periods to antibiofilm agents.

In summary, the results of the MBEC assay has shown that NAC enhances biofilm eradication and lower concentrations of rifampicin and clindamycin might be needed in the middle ear if used with pH - neutralised NAC. If this work were to be taken further, it may be useful to perform further MBEC assays working on incremental concentrations of pH - neutralised NAC from 0.5 mg / mL to 5 mg / mL against an incremental concentration gradient of rifampicin and clindamycin between 0 MIC and 100 MIC. This would be to examine the lowest concentrations of both NAC and antibiotics that produce the greatest synergy. It may also be useful to test other bacterial species common in the middle ear using the same assay given that the proof of concept has worked.

Section 8. Discussion

With the realisation that OME has a huge burden on hearing and disability in children affected, causing irreversible changes to the middle ear in chronic cases, the project set out to achieve an advancement of the current state of affairs in the management of this condition. The current treatment for glue ear is far from ideal, with ventilation tube insertion resulting in a quarter of patients requiring repeat surgery and a subset of those go on to develop more chronic problems later in life. As with other areas of medicine, advancements in the scientific arena take a while to materialise to real life patient benefits. It is often surprising how anecdotal beliefs in medicine remain unchallenged for decades even after evidence to the contrary is presented. This may be down to a number of factors: doctors are human beings and are susceptible to all the psychological dynamics that affect decision making such as cultural beliefs, pre-set ideas, previous teachings, other colleagues' ideas and thoughts, and personality barriers in analysing one's own ways.

There are other practical barriers to the scientific translation process to consider such as availability of resources, poor research training in the medical field, and clinical commitments that restrict clinicians from performing good quality research. There is also lapse between the availability of the evidence and the formation of treatment recommendations. Finally, when recommendations are published these need to be accepted within a particular specialty. Often by the time a particular idea becomes the norm, there is evidence to question its validity. Here we revisit the rationale of this research project and how this fits in with the overall picture in the management of OME.

8.1 Rationale for researching new glue ear treatment

To appreciate why the research team continues to expend time, energy and resources on finding a better treatment of OME one needs only to look at how children are affected in the critically developmental part of their lives when they are learning to speak, and how parents of those children suffer with anxiety and worry as a consequence. The personal aspects of this condition unfortunately cannot be measured as easily as the pathophysiological consequences. In order to provide a meaningful analysis of the results and how this has affected the overall goals of the research team it is important to revisit some of the evidence presented in the literature review. The high prevalence of OME affecting 80% of preschool children is not the cause of concern because the vast majority - 95% - resolve within one year (Zielhuis et al., 1990). However 5% of the those 80% becomes substantial when considering that in the year from 2009 to 2010 there were 33,920 operations to drain middle ear fluid in children below 14 years of age in England alone (hospital episode statistics-HES online database). Patient selection has improved due to the introduction of the NICE guidelines thus avoiding operating on the 95% that are expected to resolve by watchful waiting policy. The gold standard treatment currently remains ventilation tube insertion, after which a quarter of patients need repeat procedures for recurrence (Gates *et al.*, 1987). The same group of patients are likely to suffer from otopathological sequelae of scarring and destructive changes in the middle ear secondary to a local inflammatory response with the presence of matrix metalloproteinases (MMPs) (Jennings et al., 2001), interleukins, immunoglobulins, lysozyme, lactoferrin, complement components, leukotrienes and other cytokines (Geibink *et al*., 1985, Jeep, 1990).

In order to find a better treatment for OME and reduce the high rate of recurrence following ventilation tube insertion the pathogenesis of this condition was examined in detailed in Section 1.2. Briefly, the presence of local inflammatory mediators in the middle ear causes the up - regulation of mucin genes to produce mucin peptides. Bacterial lipo-polysaccharide has been shown to cause the up-regulation of mRNA of cytokine IL-8 and the increased production of MUC5AC and MUC5B in human mucin-secreting goblet cells in vivo (Smirnova *et al*., 2003). Much of this research

was in collaboration between the Newcastle-based mucin research group and Professor Birchall, one of the senior supervisors of this project (Johnson *et al*., 1997, Smirnova *et al*., 2003, Linden *et al.*, 2008). Similar findings have been presented with other research groups using animal models (Hunter *et al*., 1999). The result of mucin up-regulation is the production of a thick effusion with hearing loss as a consequence. It may be of therapeutic potential to interrupt any one of the components in the inflammatory cascades that leads to mucin up-regulation and an effusion (Figure 1.3), but a more effective strategy would be to treat the cause of this inflammation.

Following the examination of the various potential causative aetiologies for OME in Section 1.2, evidence of a bacterial cause was found to be the most substantial and robust, in particular of a biofilm infection. Standard culture techniques of middle effusion samples showed bacterial growth in 40%, but PCR of bacterial DNA showed presence in 98% of effusion samples (Paluch-Oles *et al*., 2011). These results were comparable to results from the Nottingham BRIG group where the combination of CLSM and extended culture techniques showed the presence of live bacteria in 92% of effusions (Daniel *et al*., 2012 - b). Biofilms were also demonstrated from the mucosal biopsies of the middle ear in patients with OME (Hall-Stoodley *et al*., 2006).

If the middle ear was considered as a simple organ that responds to local inflammation by the production of excess mucin, thus causing OME, the evidence presented suggests that bacterial biofilm in the middle ear is the cause in the majority of cases. Although production of various mucins has many protective roles as discussed in Section 1.2.2 (Reddy et al., 1987), excess mucin production may also be the perfect attachment substrate and source of nutrition for bacterial biofilms. Biofilms of *Ps aeruginosa* formed on mucin-coated surfaces have been shown to develop large cellular aggregates and increased tolerance to the antibiotic tobramycin when compared with biofilms grown on glass, actin-coated, or DNA-coated surfaces (Landry *et al*., 2006). Bacteria from human dental plaque have been shown to utilise salivary MUC5B mucin as a nutrient source by proteolytic degradation (Wickström and Svensäter, 2008).

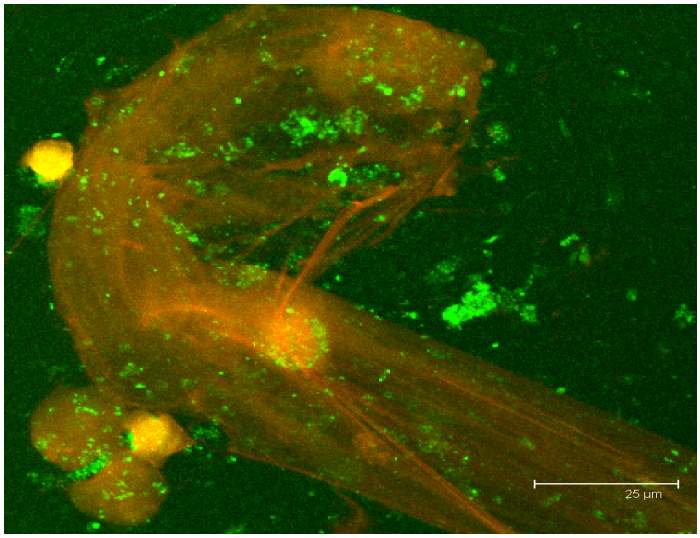


Figure 8.1 CLSM of glue ear sample stained with periodic acid schiff. Mucin strands seen red. Live / dead stain showing live bacterial biofilms in green. (Daniel *et al*., 2012 – a)

Given the advantage incurred by bacteria present in a biofilm state and additionally with the presence of mucin as an attachment substrate (Figure 8.1) and a nutrient source, the goal for this research was to find therapeutic strategies to breakdown mucin molecules and bacterial biofilm structure. By removing the attachment site for biofilms in the middle ear theoretically this would weaken their structure and reduce their recalcitrance to antibiotics. Thick mucin is the cause of hearing loss in the middle ear, and the use of NAC, a chemical with mucolytic properties, would in theory be beneficial by reducing thick effusions to thin serous fluid that can be cleared via the eustachian tube more easily as explained in Section 1.3.7. The mucolytic mechanism of action of NAC is down to its thiol group: mucin glycoprotein oligomers are depolymerised by hydrolysis of the disulphide bonds (S-S) that link mucin monomers. The resultant sulfhydryl bonds (-SH) are no longer able to crosslink and this reduces the viscosity of mucus (Sheffner, 1963). For these reasons NAC was chosen for both its mucolytic properties and its biofilm - reducing potential.

The anti-biofilm properties of NAC are attenuated when combined with antibiotics, and for this reason it was tested in combination with rifampicin and clindamycin. Rifampicin and clindamycin were chosen for knowledge of their efficacy of known bacteria present in the middle ear (Daniel *et al*., 2012 - b) and when used in combination they reduce the risk of mutational resistance (Bayston *et al*., 1989). NAC has anti-oxidant protective properties to the inner ear structures: it has been found to improve recovery of hearing following sudden sensori-neural hearing loss (Angeli *et al*., 2012). This property was thought to be beneficial to counter potential ototoxicity of antibiotic use in the middle ear.

The proposed strategy of targeting the middle ear with NAC, rifampicin and clindamycin was through local drug delivery. This can be done at the time of ventilation tube insertion where the tympanic membrane is incised, middle ear effusion suctioned, the drugs can then be delivered into the middle ear following which a ventilation tube can be inserted. It is expected that eradication of middle ear biofilms and breakdown of mucin in the middle ear would reduce the recurrence rate of OME following ventilation tube insertion. Local drug delivery was thought to be most beneficial with the knowledge that biofilms require high doses of antibiotics for eradication, typically 100 to 1000MIC (Daniel *et al*., 2012 – a). This cannot be achieved through systemic administration as severe toxicity to various organs in the body would ensue at these concentrations. The drug delivery agent has been developed and tested by Matija Daniel (PhD student) and the BRIG team. It consists of a 3mm diameter biodegradable polymer, poly-lactic-co-glycolic acid (PLGA) shown in Figure 8.2. Drugs can be impregnated by adding to the co-polymer before sintering and they are released over a 3-week period. The PLGA scaffold is biodegradable and can theoretically be absorbed by the middle ear mucosa. Building on the previous achievement of the research team with the PLGA drug delivery scaffold, the aim of this project was to explore the use of NAC as a potential therapeutic agent for the treatment of OME.

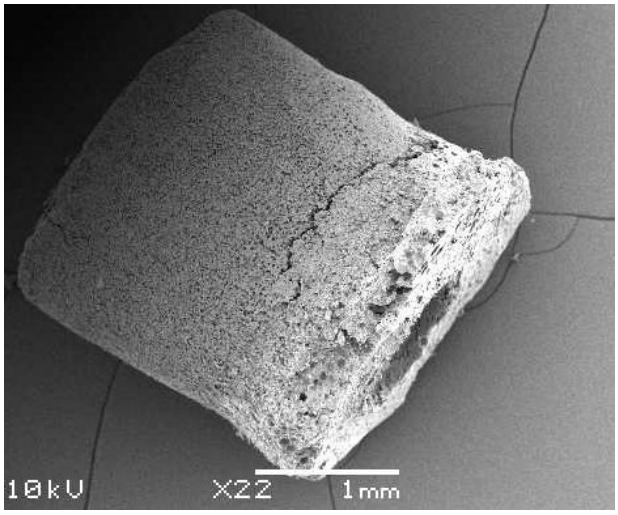


Figure 8.2 Middle ear drug delivery scaffold made of biodegradable poly-lactic-co-glycolic acid (PLGA)

NAC has anti-biofilm, mucolytic, and anti-oxidant effects that are beneficial in the context of OME, but the anti-biofilm potential was thought to be the most important to investigate and was the focus of this project. This was from knowledge of the difficulties when treating biofilm-related infections, especially once mature biofilm establishes in a certain environment. This certainly reflects the clinical picture of chronic cases of OME resistant to multiple ventilation tube insertions. If NAC can potentiate the action of antibiotics against biofilm, this would increase the chance of success in eradication and also reduce the antibiotic concentrations required. It may also reduce the potential for ototoxicity from use of high antibiotic doses in the middle ear. The effect of NAC against live biofilm was initially observed for proof of concept and to confirm findings of other papers in the literature. Following this, consideration of the mechanism of action was needed to elucidate how NAC was reducing biofilm. This was felt to be important from an academic and research standpoint, as most of the studies identified in the literature used final outcomes of NAC addition to biofilm, but did not look at the mechanism of action. The effect of NAC addition to extra-cellular slime was examined using the alcian blue GAG assay, and cellulose acetate electrophoresis. More importantly for the clinical aim of the project was a closer look at the effect of NAC in combination with rifampicin and clindamycin. The purpose of this was to find the lowest concentration of NAC, rifampicin, and clindamycin needed to eradicate biofilm using a high throughput micro-titre well MBEC assay.

8.2 Interpretation of results and significance in light of previous research

The bacteria used for this study were isolated from middle ear effusion samples of children undergoing ventilation tube insertion for OME. The samples had been stored in cryoprotectant at -22°C for 4 years prior to use in the project. Unfortunately many of the isolates were either dead, or were contaminated with other bacteria than originally documented. This had implications on the availability of bacterial species to work with. This may have been due to repeat freezing and thawing whenever the cryo-samples were removed from the freezer and returned. Alternatively it may have been due to previous poor techniques when isolating the samples resulting in either a small bacterial quantity frozen or contamination with other species. All the *H influenzae* samples in the storage pool were dead reflecting the conditions it requires for survival. A study on the storage conditions of *H influenzae* revealed that the concentration of the initial inoculum affected survival when stored at -20°C, but at -70°C the initial concentration did not affect survival (Aulet de Saab *et al*., 2001). This implied that the viability of bacteria reduces over time at -20°C faster than -70°C, and with 4 years of storage time in our study it is not surprising that the bacteria were dead by the time they were cultured.

In an initial attempt to use bacteria that resemble bacterial isolates of middle ear effusion samples (Giebink *et al.*, 1982, Stanievich *et al.*, 1981, Daniel *et al*., 2012 – b) when performing biofilm experiments, an isolate was chosen from each of the following species: *S epidermidis*, *S aureus*, *M catarrhalis*, *S pneumoniae*. A wild strain of *H influenzae* was obtained from a child with an eye infection due to the death of bacteria from the storage pool. It soon became apparent that it was practically impossible given the time frame of the project and the resources to replicate each experiment five times with each one of those bacteria. Prior to this realisation, the crystal violet biofilm assay was used to quantify the ability of each of these bacteria to produce biofilm.

Crystal violet assay

Although the results of the crystal violet assay have been presented in Section 4, it was felt that those were unreliable due to many issues with the reproducibility of the assay. There was considerable variation in the results with minimal changes to the PBS washing step, the method of removal of the rinsed fluid, the batch of crystal violet used (data not presented in Section 4), and the type of microtitre well used. An important disadvantage to the basic principle of the technique is that it is a semi-quantification of the biofilm based on an optical density reading of the concentration of crystal violet bound to what is presumed to be biofilm. There is no way to confirm that the actual material measured was definitely biofilm and not waste products of bacteria attached to the wells. Also the assay does not give a final cell count of the bacteria in the biofilm, unlike the cell viability technique and the MBEC assay described in Section 5 and 7 respectively both of which give CFU readings. After following the recommendations by Stepanovic *et al*., (2000) to correct these variabilities the crystal violet assay gave two different readings on repeat of the same experiment despite using the same technique. To illustrate this variability, Fredheim *et al*., 2009 showed that using the Stepanovic technique 18% of *S haemolyticus* isolates produced biofilm. With a simple modification by including the air-liquid interface of the well in the analyses 74% of the isolates were found to be biofilm producers. The visual analysis of the wells from this project in Section 4 showed there was a considerable amount of crystal violet attached on the wells in the air-liquid interface and therefore a similar modification was performed to include this in the analysis. There was a real risk however that this merely represents by-products of bacteria floating to the air-liquid interface at the top of the wells. We therefore take the results of the Section 4 with great scepticism.

Silicone disc biofilm assay

As it was not possible to accurately identify strong biofilm producers using the crystal violet assay, and given the practical issues of testing five bacteria repeatedly, a *S aureus* isolate from a middle effusion sample with previously proven biofilm producing capability (Daniel *et al*., 2012 – a) was used for the rest of the assays. On performing a proof of concept experiment using the silicone disc biofilm assay, after only 24 hours NAC was found to completely inhibit bacterial growth in biofilm and eradicate planktonic bacteria. This was later found to be largely due to an extremely low untested pH of 1.4. A re-examination of the MIC's for NAC showed a minimum bactericidal concentration of non-neutralised NAC was as low as 15mg/mL, in contrast to no bactericidal effect of neutralised NAC even up to 200mg/mL, and the MIC value not being found.

It was interesting to compare these results to other studies of NAC. NAC at 80mg/mL has been shown to have an antimicrobial effect against biofilm-associated MRSA, MRSE, and *Klebsiella pneumoniae* (Aslam *et al*., 2007). This was not the case for MSSE, VRE, and *Acinetobacter baumannii*. There was variation of the NAC effects depending on the bacterial species of the biofilm. There was no mention in the article of whether the pH of NAC was accounted for, and it is difficult to know whether their results may have been pH-related similar to that observed in Section 5. Certainly in the MBEC assay there was no effect from the addition of neutralised NAC on live biofilm when used on its own.

MBEC assay

Although the experiments in this project were presented according to the sequence they were performed, for purposes of discussion it was more logical to discuss the effects of neutralised NAC observed on live biofilm from Section 7 before embarking on the postulated mechanisms of action tested in Section 6. Results from the MBEC assay showed that neutralised NAC at 5mg/mL with rifampicin and clindamycin together at 100MIC eradicated biofilm after a one-week exposure when compared to rifampicin and clindamycin alone at 100 MIC that failed to eradicate biofilm with a CFU quantity of 3.53×10^8. This was in contrast to non-pH - neutralised NAC that eradicated biofilm with a concentration ten times less, 0.5mg/mL, when used with rifampicin and clindamycin together at 100MIC after a one-week exposure. Similarly, tigecycline 1000MIC with NAC 80mg/mL has been found to be synergistic after 4 hours of incubation for MRSA, and 12 hours for MRSE (Aslam *et al*., 2007). Similar results were reported for the combination of fosfomycin and NAC against *E coli*, showing synergy with the use of antibiotics with NAC (Marchese *et al*., 2003). Again, there was no mention of pH measurements for the NAC and it is not known if their results were pH related.

A study on the effects of NAC presence on biofilm growth of *S epidermidis* showed that at a concentration of 8 mg/mL NAC completely inhibited growth of four strains, and reduced growth by more than 50% of six strains (Perez-Giraldo *et al*., 1997). There was no mention of pH adjustment in the paper. This is comparable to results from Section 5, with non-neutralised NAC at 50mg/mL inhibiting biofilm growth after just 24 hours' exposure. Also similar to the results from Section 6.4, the MBC of non-neutralised NAC was 15mg/mL. The methodology for growing biofilms were not comparable between the various papers mentioned or compared to this project, and nor were the technique for measuring biofilm. Nevertheless, the concentrations quoted indicate that none of the studies neutralised NAC prior to testing, because the results obtained resemble those of this study when non-neutralised NAC was used.

Two studies were identified that did neutralise NAC prior to use in combination with antibiotics against biofilms (Venkatesh *et al*., 2009, Zhao and Liu, 2010). Comparisons of our results with these studies are therefore more meaningful. The first looked at the effect of neutralised NAC on *Ps aeruginosa* biofilms (Zhao and Liu, 2010). Interestingly, the NAC MIC's for twenty isolates of *Ps aeruginosa* were between 10 and 40mg/mL in contrast to this study where the MIC of *S aureus* was not reached even at an NAC concentration of 200 mg / mL. It may be that *Ps aeruginosa* was more sensitive to NAC than *S aureus*. The paper did mention that two of the isolates had MIC's > 40mg/mL without mentioning how high it was. It therefore appears that sensitivity to NAC depends on the bacterium used. This was an important reason to repeat the MBEC assay in this study with the five bacterial species initially intended that resemble those found in middle ear effusions. However due to time constraints this was not possible. The paper demonstrated that NAC could detach mature biofilm in proportion to the concentration used. Silicone discs similar to those in this study in Section 5 were placed in a 24 well plate and biofilms allowed to mature for 6 days prior to adding combinations of NAC (0 to 2.5mg/mL) and ciprofloxacin (0 to 8 MIC) in a checkerboard format for 24 hours at 37°C, then viable cell counts were performed similar to methodology used in Section 5. Synergy was seen with NAC at 0.5mg/mL in combination with ½ MIC of ciprofloxacin. All other combinations of NAC/ciprofloxacin showed reduction in biofilm. These results strongly support our findings showing the potent effect of NAC with antibiotics on biofilm. The paper differs in that we were unable to show an effect on biofilm with the use of neutralised NAC on its own.

Another study that neutralised NAC (Venkatesh *et al*., 2009) showed that NAC at 8mg/mL reduced biofilm thickness (μm) and biomass (μm^3), measured by CLSM, of *S epidermidis* after 24 hours' exposure when compared to control. The study also examined NAC for synergy with vancomycin and nafcillin against biofilms of *S epidermidis* in 8 x 8 checkerboard format using XTT reduction assay. To check for synergistic drug combinations, the study used the median effects method to calculate combination indices (CI). Multiple steps in the calculation were described in the study using computer software. NAC was found to be synergistic with nafcillin and vancomycin separately at multiple concentrations ratios for the three isolates of *S epidermidis* tested. The calculations described in the paper make it difficult to work out what concentrations were used because the results were presented as ratios.

The two studies described have so far shown reduction in biofilm using neutralised NAC alone on *S epidermidis* and *Ps aeruginosa* (Venkatesh *et al*., 2009, Zhao and Liu, 2010). This calls into question why our study was unable to demonstrate this using the MBEC assay. It may be that the specific *S aureus* isolate used was particularly recalcitrant to NAC, or a possible difference in the methodology of the assays. Given that NAC was used up to a concentration of 50mg/mL, one would expect to have seen some reduction when comparing with the other studies mentioned. To investigate this further it would be important to repeat the MBEC assay on various other bacterial species and preferably to the same bacterial isolates used in those studies.

Mechanism of action studies

Many of the studies described did not differentiate the effects of NAC on bacteria from bacterial ECS. This may be satisfactory from a clinical standpoint, as it may be argued that the mechanism of action does not matter as long as a drug performs a beneficial action. From an academic and biofilm research standpoint however, this is not satisfactory because it does not provide answers to many questions posed following their observations. We designed separate assays to test NAC effect on biofilm using the MBEC assay previously described, and on ECS separately. A method for purification of ECS from bacteria was adopted from Bayston & Rogers (1990). It was observed that the addition of NAC to ECS caused instant turbidity. Following investigation of this, NAC was found to have a pH of 1.4 at the concentration of 50mg/mL used. On neutralization of NAC pH this turbidity was no longer observed. Similar turbidity was observed on addition of acetic acid or hydrochloric acid to ECS. When sodium hydroxide was added to reverse pH, the turbidity disappeared. It was therefore concluded that ECS was insoluble at a low pH. The reason postulated was that extra H^+ ions on the ECS molecule caused a change in the molecular shape and this caused hydrophobicity. The effect of this on live biofilm was observed in the silicone biofilm disc assay where non-neutralised NAC caused complete inhibition of biofilm growth after 24 hours' exposure and biofilm eradication after 7 days. The potential significance was that acid could be used as biofilm - reducing agent whenever pH is not an issue. Unfortunately, for the purposes of middle ear biofilms, a low pH in the middle ear would potentially have devastating corrosive effects in one of the most sensitive and protected areas in the body.

The alcian blue GAG assay measured quantity of ECS. Following the addition of non-neutralised NAC, there was a reduction in the total ECS quantity. When neutralized NAC was used, there was no reduction in the total ECS. This reduction was due to the precipitation of ECS secondary to low pH that was washed away in the rinsing step of the assay. This did not give us information on whether neutralized NAC had any effect on ECS molecular structure, and the cellulose acetate electrophoresis assay was therefore employed. Both neutralised and non-neutralised NAC showed a significant change in the electrophoresis pattern following addition

to ECS (Figure 6.3 and 6.7). This pattern change was not replicable on use of acetic acid or hydrochloric acid (Figure 6.5). From the analysis of the patterns there appears to have been a reduction in the stain quantity, as well as a change to the pattern of the stain.

A study of NAC mechanism of action was identified where the effects of neutralized NAC on bacteria and on bacterial ECS were investigated separately (Olofsson *et al*., 2003), unlike previous studies that did not make this distinction. The study found that NAC at a concentration of 2mg/mL inhibited growth of *Acinetobacter baumannii, Acinetobacter lwoffii, Bacillus sp., Enterobacter cloacae, Klebsiella pneumoniae, Pseudomonas mendocina, and Staphylococcus warneri*. This was largely contrasting our results where the MIC was not reached at neutral NAC of 200mg/mL for *S aureus*. NAC was added to those bacteria in static phase growth - starvation, and was shown to decrease the amount of ECS production on average by 58% when added at a concentration of 0.25mg/mL. They were unable to show any degradation of the polysaccharides by addition of NAC to purified ECS. This is similar to our results from the alcian blue GAG assay showing no degradation in the total measured ECS following addition of neutralized NAC. Both the Olofsson study and this study used similar principles for purification and measurement of ECS with minor differences that were not felt to have affected the outcome: in the Oloffson study stationary phase grown bacteria were separated from ECS by 30 seconds of sonication at 10% strength then centrifugation for 10 minutes at 950 x g, in this study the samples were not sonicated but centrifuged for a longer period of 15 minutes at 7500 rpm and 4°C. Methods for measuring ECS were also similar: Oloffson study used tryptophan to bind carbohydrates and measured spectro-photometrically, in this study alcian blue was used to bind carbohydrates and measured spectro-photometrically. The Oloffson study also demonstrated that NAC at a concentration of 0.5mg/mL detached biofilm from stainless steel surface, and was able to inhibit the initial adherence of bacteria to stainless steel.

A further published report by the same research team examined the effects of coating NAC on a stainless steel and a polystyrene surface (Olofsson *et al*., 2005). In essence, NAC was found to increase the wettability of the surface attached. NAC-coating increased the visco-elastic properties of the surface resulting in a more flexible binding between cells and the surface. This resulted in a smaller number of irreversibly adhered bacteria, as a larger number of bacteria were washed off. The cell surface hydrophobicity (CSH) of a bacterium was found to be an important factor in the affinity to a hydrophobic surface. *B cereus* has hydrophobic spores that help pierce through to the surface against the anti-adhesive forces. Therefore a decrease in the hydrophobicity of the surface reduces this effect. Our study did not specifically examine the effects of NAC on adhesion and therefore this study provides an interesting insight to an important anti-adhesive property for this potentially therapeutic drug.

8.3 Research hypothesis

The initial proposed hypothesis was that NAC may enhance biofilm disruption and lower the dose of antibiotic needed to eradicate middle ear biofilm. After adjusting for the initial pH related effects, the MBEC assay demonstrated this hypothesis was true. NAC at a concentration of 5mg/mL with rifampicin and clindamycin at 100 MIC eradicated 4-day mature biofilm after 1 week of exposure. NAC at a lower dose of 0.5mg/mL and combinations of 100, 1000, and even 10000MIC of rifampicin and clindamycin together did not eradicate biofilm after 1 week exposure. This seems to agree with other studies in the literature discussed in previous section 8.2. In addition to the research hypothesis, this study has demonstrated a direct NAC effect on purified ECS using the cellulose acetate electrophoresis. This has added a novel insight into the mechanism of action of NAC that has not previously been performed following a literature review.

8.4 Research limitations and future suggestions

Limitations of assays

The main advantage of the crystal violet assay was that it employs a high throughput technique to quickly screen for biofilm production capability of various bacterial species in one assay run. The disadvantages and unreliability considerably limited the use of this assay to make any meaningful conclusions about the biofilm - producing ability of the bacterial isolates. The reasons for the unreliability have been discussed in detail previously. The silicone disc biofilm model based on the viable cell count technique was initially used to show that NAC reduces biofilms. As an assay this was found to be very robust with reproducible data in the method development part of this project. Thus the results could be taken with high degree of confidence. The main disadvantage of this assay was that it was very labour intensive. With the increasing need to test more concentrations and adjust for various factors such as comparison of neutralised versus non-neutralised NAC this was practically impossible given the time frame and the resources available because it required the use of a large quantity of agar plates and took approximately six weeks to complete one assay run. If time and resources allowed, it may have been preferable to perform the synergy testing using the checkerboard with the silicone biofilm disc method.

The MBEC assay was chosen as it was a high throughput technique and combined the basic technique of the viable cell count method in a 96 well format. This had the advantages of the silicone biofilm disc, and the ability to test an increased number of combinations with the ample wells available. The assay took approximately three weeks to complete one run, but the number of combinations tested was about 4 times as many for the same effort. The results from the MBEC assay may not have been comparable to the silicone disc due to various differences in processing techniques and the maturity of biofilms tested, these were summarised in Section 7.4. Similar to the crystal violet assay, there were multiple potential steps in the MBEC assay that could introduce variability in results if two different users introduced small

modifications without realising. Due to this reason the methodology was adhered to strictly and the steps of the assay were documented clearly.

When testing for the effects of NAC on ECS, the alcian blue GAG assay measured the total amount of ECS. The assay did not give an indication of the composition such as the quantity of proteins, polysaccharides, and DNA in the ECS. Further experiments may be adopted for future testing if the time and resources allowed, as this would have been very helpful when looking at how NAC may be affecting the composition of some elements that make up ECS. The assay did give an overall impression that there was a major reduction in ECS such as that seen when adding acid due to precipitation. Due to the size of the large PIA molecule, it would not have been possible to use SDS-PAGE electrophoresis, as the large PIA molecule would not have travelled through the medium. Cellulose acetate paper was therefore used. The major advantage was that it allowed the measurement of large molecules, but as can be appreciated from the pictures in Section 6.8, the quality of the stains was not comparable to the clear bands obtained in Western blotting that uses SDS PAGE electrophoresis to separate proteins of various molecular sizes. Because large molecules were being measured, it may have been useful to have more controls with known molecular size so as to compare the stains obtained.

Future considerations

Although NAC was chosen due its mucin - breaking potential, the evidence for this potentially therapeutic property in the literature was overwhelming and was not investigated. The time of the project also did not allow such experiments to be performed. However, it would have been of great benefit to compare the effects NAC on biofilms with the presence of mucin. This may represents closer *in vivo* conditions to that in the middle ear. Biofilms are known to use mucus as a substrate for attachment and for nutrients. The addition of NAC to biofilms grown on mucin may have had multiple actions, both on the biofilm, and on the mucin, thus employing a multiple - pronged attack.

The observed effect of low pH on biofilm was an interesting observation. For future it may be interesting to see what the effects of various acids on biofilms at different concentrations are. Although the use of acid is restricted in the medical field, this may be of benefit to biofilms encountered in the industry.

The effect of NAC on biofilm growth was not tested. An additional assay to perform would have been to grow biofilms in the presence and absence of NAC at multiple concentrations. This would have demonstrated whether NAC inhibits growth of biofilm. As *S aureus* was used for all the assays, there is an assumption that the results will be similar for the other commonly grown bacteria in the middle ear. Without repeating the same experiments for the other *S epidermidis*, *M catarrhalis*, *S pneumoniae*, and *H influenzae*, the generalisability of the assays was restricted. In addition, the particular *S aureus* isolate used may have different properties from other isolates and therefore the results may have been more robust if the same effects were demonstrated on multiple isolates. Despite the limitations suggested, the results indicate a clear benefit for the use of NAC with antibiotics to eradicate biofilm in the middle ear.

8.5 Future direction of research

The work of this project has demonstrated that NAC is a real therapeutic potential for the treatment of OME. Its antibiofilm properties are potentiated when used in combination with rifampicin and clindamycin. The effects on ECS the major component of biofilm were also demonstrated. NAC has also has mucolytic and anti-oxidant properties that would be beneficial for use in the middle ear. The optimum antibiofilm concentrations demonstrated were 5mg/mL of NAC with 100MIC of rifampicin and clindamycin together. The next step would be to incorporate these concentrations into the biodegradable PLGA pellet that has previously been developed. The release profile of rifampicin and clindamycin by the biodegradable pellet have been studied, and it was found that 1000MIC of those antibiotics need to be released for three weeks for biofilm eradication to occur (Daniel *et al*., 2012 - a). In the light of our results, only one week of release of 100MIC rifampicin and clindamycin with 5mg/mL NAC was needed. This reduces the potential ototoxic effects of the antibiotics, as the duration and concentration has reduced to achieve the same goal. The full potential of NAC however was not demonstrated because biofilms grown on mucin are theoretically more susceptible to NAC for reasons discussed. Therefore to make use of these results, further experiments of incorporating NAC into PLGA and studying its release profile are needed.

There are a few obstacles to be overcome. NAC neutralisation in solution was performed by titrating sodium hydroxide until pH of 7 was reached. This needs modification if the neutralised NAC is to be incorporated into the PLGA scaffold. It may be possible to neutralise NAC in solution as described then boil this to crystallisation. Whether this affects the final potency and molecular structure of NAC is yet to be established, though this is not expected. The neutralised NAC crystals can then be mixed with rifampicin and clindamycin to make the PLGA scaffold. Following the incorporation into the PLGA scaffold, it would be imperative to also study its effect on biofilm. Although NAC in solution was shown to potentiate biofilm breakdown when used with antibiotics, this needs to be replicated with the use of the scaffold. Currently the research team has initiated experiments to incorporate NAC into the PLGA.

After this step, ototoxicity studies need to be performed to ascertain at what levels do NAC, rifampicin and clindamycin become ototoxic. Ototoxicity can be in the form of damage to vestibular apparatus responsible for balance through diffusion across the oval and round window therefore causing potentially debilitating vertigo and imbalance. It can also potentially cause cochlear toxicity causing irreversible sensori-neural hearing loss, also a devastating outcome for children in the early stages of their lives. So far there are no data to suggest that rifampicin and clindamycin are ototoxic, but this needs to be confirmed *in vivo*. NAC has been shown to have a vestibulo-cochlear protective role, and its use in the middle ear is not worrisome from a toxicological point of view. In fact it is hoped to counter any toxic effects of antibiotics. Oral NAC and oral steroids reduce the amount of hearing loss in sudden sensori-neural hearing loss when compared to oral steroids alone (Angeli *et al*., 2012). The research team has currently started preparations for work with rodents to answer the above questions posed. Once this is complete the plan would be to perform a randomised clinical trial to test the PLGA drug delivery scaffolds in the middle ear of children with OME.

Section 9. Conclusion

Main points that have emerged

Bacterial biofilms, mucins, and their interactions have emerged as important players in the development of glue ear. Biofilms have been extensively researched in the industrial fields as they cause major problems to pipeline and various other places. Only over the last three decades has the role of biofilms in chronic infections become apparent. Instead of re-inventing the wheel, it makes sense to learn from the lessons learned from the industry about biofilms. Making use of this knowledge we were able to apply similar principles to understand how biofilms behave, how they structure themselves, and how it may be possible to break them down. To find a better treatment for glue ear, one has to look at all the potential aetiological factors that may influence outcome. To think only from a biofilm standpoint it is possible to overlook other important factors that lead to glue ear. Humans are complex beings and are affected by a wide array of environmental factors. It may be impossible to determine all the factors that are at play in the development of disease, however for therapeutic purposes targeting the most suspicious culprits would be a good strategy.

Biofilms are highly recalcitrant to antibiotics. A strategy developed was to break down the biofilm structure using NAC to increase the likelihood of eradication with antibiotics. NAC also has mucolytic properties by breaking the di-sulphide bonds between the mucin molecules by virtue of its thiol group. When one considers that biofilms in the middle ear use mucus as a substrate for attachment, growth and nutrition, one can only begin to realise the potential benefits of NAC. We have shown the potent effects of NAC when combined with rifampicin and clindamycin against mature biofilms, in addition to its effect on ECS, the major component of biofilm. This information has allowed the research to team to continue in this direction by incorporating NAC, rifampicin, and clindamycin into a PLGA biodegradable polymer that can be inserted into the middle ear at the time of ventilation tube insertion to eradicate biofilms. By targeting the underlying cause of glue ear, it is hoped that this novel intervention would result in a better outcome

from the currently high rate of recurrence following ventilation tube insertion. If this goal were achieved then that would provide further evidence in support of the bacterial biofilm theory. Also the benefits for patients affected are not measurable. The vision of the research group would be that patients with glue ear would have only one anaesthetic with this novel technique that results in complete resolution thus avoiding long-term middle ear destructive consequences of the chronic inflammatory process of OME. To achieve this vision may not be possible, but to get close would be a huge milestone.

Other possibilities

The results of this work are transferrable to many arenas in ENT and other avenues in medicine. Biofilms are increasingly being recognised as causing many indolent chronic conditions. There is evidence to suggest that biofilms have a role in chronic rhino-sinusitis, recurrent tonsillitis, mastoids in chronic otitis media, cholesteatoma, and chronic laryngitis (Vlastarakos *et al*., 2007, Post *et al*., 2007, Kinnari *et al*., 2012, Lampikoski *et al*., 2012). The therapeutic possibilities are transferrable. The potential for ototoxicity from therapeutic drugs in the middle ear is not present in recurrent sinusitis or chronic laryngitis, and therapy with a combination of antibiotics and mucolytics can be used for those infections if proven to be of biofilm aetiology. The drug delivery may however need to be adjusted, such as an inhaled aerosol or mouth gargles containing rifampicin and clindamycin with NAC. Also the antibiotic choice may need to be tailored given the prevalent bacterial biofilms overlying the mucosa of the area of interest. For chronic rhinosinusitis there is evidence that nasal douches with salt water improve symptoms (Harvey *et al*., 2007). A similar regimen with salt, water, and NAC may be offered to reduce the thick mucus secretions in the nasal cavities and reduce the substrate for biofilm attachment. This would be a potential avenue for future research.

The PLGA scaffold has been developed as a drug delivery carrier into the middle ear. This would need a general anaesthetic and an incision of the tympanic membrane to deliver the PLGA. As research into drug delivery techniques advance, this step may be avoided. Various techniques have been used for drug delivery to the middle ear (Hoskison *et al*., 2013), but the method of interest was delivery through

the tympanic membrane without the need for incision. This can be done using nano-particles ionically charged by passing a current, and then moved across the tympanic membrane by applying a magnetic field into the middle ear. This can potentially be done in the outpatient setting however this may not be practically possible with children that are not cooperative. It is not known what drugs are suitable for this technique, and there may be a change to the properties of the drugs following ionisation. Nevertheless it is an interesting new avenue for research potential.

The basic principles of biofilms will always apply, but the therapeutic possibilities are very dynamic thus making research into biofilm-related infections an ever-growing field with exciting new possibilities. To offer patients the best care possible, the medical field needs to be in tune with the new scientific advances and utilising those novel treatments for medical conditions. The importance of collaborating with scientists, pharmacologists, microbiologists, physicians, and the pharmaceutical industry cannot be over-emphasised if these goals are to be realised.

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