Thesis

ANTIBACTERIAL PROPERTIES OF NOVEL DENTAL COMPOSITES FOR PAEDIATRIC DENTISTRY

Submitted by

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Declaration

I, Nikolaos Lygidakis, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: _____ Date: _____

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Abstract

Aim

To assess the antibacterial properties of novel dental composite formulations containing the antibacterial polylysine and varying type or amounts of monomer/glass/calcium phosphate.

Methods

Minimum inhibitory/bactericidal concentrations of polylysine against *Streptococcus mutans* UA159 were determined. The antibacterial activity of composite discs with polylysine was determined by immersing the discs into a suspension of *S. mutans* and carrying out bacterial counts. All the results were compared with commercial materials. Mass and volume change of the material as well as polylysine release were determined over time and compared for multiple formulations containing polylysine. Bacterial growth was visualised on the discs using LIVE/DEAD staining with confocal microscopy and using scanning electron microscopy.

Results

The addition of a minimum 1% polylysine to the novel formulations inhibited bacterial growth at low inoculum density and the addition of a minimum 2% polylysine inhibited bacterial growth at all inoculum densities in air. In an atmosphere of air enriched with 5% carbon dioxide and in the presence of sucrose there was a bacteriostatic effect with 5% polylysine addition. None of the commercial materials showed any antibacterial properties. Increasing the amount of polylysine in the novel composite formulations increased mass change over two months and increased polylysine release over three weeks. Volume was not significantly affected. Using SEM, bacterial growth was seen on composite discs after 4 days incubation in a suspension of *S. mutans* at 37^{o}C in air with 5% carbon dioxide. It appeared that a biofilm was formed under these conditions for all formulations and commercial materials whereas in air, there was minimal growth. Using confocal microscopy an increase in dead bacteria was seen as the polylysine concentration increased in both air and in air with 5% CO_2.

Conclusion

Novel composites with added polylysine are capable of reducing the load of *Streptococcus mutans*. These above experimental composites have novel characteristics that make them more suitable for minimally invasive tooth restorations.

Key words: polylysine, composite, restoration, antibacterial properties

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List of Abbreviations

4-META	4-methacryloxyethyl trimellitate anhydride
ACP	amorphous calcium phosphate
AEP	acquired enamel epithelium
BHI	brain heart infusion
Bis-EMA	ethoxylated bisphenol-A dimethacrylate
Bis-GMA	bisphenol-A glycidyl methacrylate
BPA	bisphenol-A
CaP	calcium phosphate
CHX	chlorhexidine
CQ	camphorquinone
CV	crystal violet
DCP	dicalcium phosphate
DMAEMA	2-(*N,N*-dimethylamino)ethyl methacrylate
DMAHDM	dimethylaminohexadecyl methacrylate
DHEPT	N,N-dihydroxyethyl-p-toluidine

DMFT	decayed, missing, filled teeth
DMPT	*N,N*-dimethyl-*p*-toluidine
DW	deionised water
ECC	early childhood caries
EPL	polylysine
EPS	extracellular polysaccharides
FDA	food and drugs administration
GIC	glass ionomer cement
GTF	glucosyltransferases
HA	hydroxyapatite
HEMA	2-hydroxyethyl methacrylate
HPLC	high performance liquid chromatography
MBC	minimum inhibitory concentration
MCPM	monocalcium phosphate monohydrate
MIC	minimum bactericidal concentration
NCCI	non cavitated carious lesion
OD	optical density
PLR	powder to liquid ratio
PPGDMA	polypropylene glycol dimethacrylate
RMGIC	resin modified glass ionomer cement
SEM	scanning electron microscopy
TB	trypan blue
TCP	tricalcium phosphate
TEGDMA	triethyleneglycol dimethacrylate
UDMA	urethane dimethacrylate
WHO	world Health Organisation

List of symbols

a	absorbance
v	volume
v_0	initial volume
v_t	total sample volume
m	mass
m_0	initial mass
m_{c1}	mass of specimen in air
m_{c2}	mass of specimen in buoyancy medium
m_t	total sample mass
$m_{EPLsample}$	mass of polylysine in the sample
$m_{EPLsolution}$	mass of polylysine in solution
ρ	density
ρ_0	density of buoyancy medium
P	powder to liquid ratio
D	Diffusion coefficient
x	mass fraction of EPL in the powder

1. Chapter One: Literature Review

1.1 Caries

1.1.1 Definition

Dental caries is caused by the demineralisation of hydroxyapatite by acids coming from bacterial fermentation of sugars. These bacteria reside in the dental plaque on the tooth surface (Loesche, 1996).

Dental caries is one of the most common diseases in the world. It is estimated to affect 31% of adults in the United Kingdom and the National Health Service spends approximately 5.8 billion pounds per year on restoring teeth at the dentist (Steele & O ’Sullivan, 2009).

1.1.2 Epidemiology

Oral health has significantly improved since the 1970s. Generally, this is due to the availability of fluoride in toothpaste but also in water, mouthwash, tablets and other. However, almost a third (31%) of 5 years old children in the UK still have caries on their primary teeth and the ones who are affected will have at least three decayed teeth. Dental caries is the most common reason for a young child to have a general anaesthetic, and caries is a disease that can be prevented through oral hygiene and tooth brushing (Reena, 2012). One of the most recent epidemiological studies in the UK was done in 2013. Nearly half (42%) of 15 year olds and a third of 12 year olds has had previous caries experience in the permanent dentition (Pitts & Chadwick, 2013). This has been reduced by approximately 10 percent since 2003 when the previous survey was done. Also in 2013, and in the younger population, a third of 5 year old and almost half of 8 year olds has had previous decay in the primary dentition. Socioeconomic level also plays a role and children from lower income families are more likely to have dental caries (Pitts & Chadwick, 2013). Although the above numbers seem significant, if these are compared with 1973, when the first children dental health survey was done, it is clear that the reduction is huge. Since 1973 approximately 70,000 children between five to fifteen years old have been involved in the child dental health survey. In the UK, caries prevalence has been reduced from 72% to 41% in 5 year olds and from 97% to 46% in 15 years olds (Murray et al., 2015). The table below shows the reduction of dental caries in recent years.

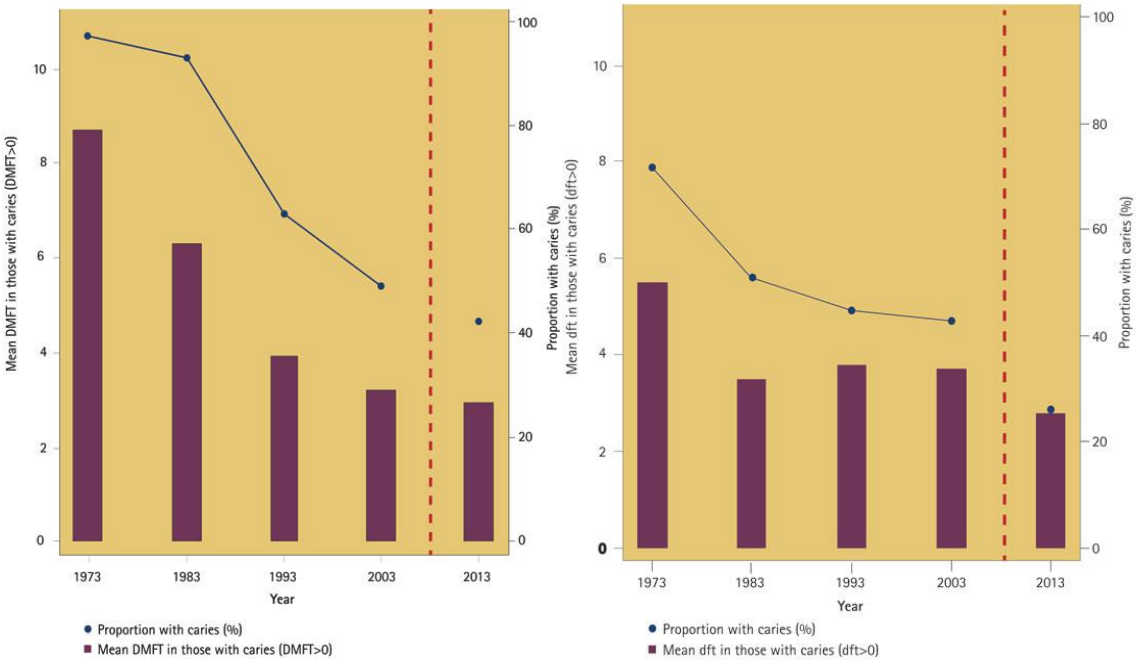

Figure 1-1: DMFT and proportion with caries in 15 (Left) and 5 (Right) year old children in UK (Murray et al., 2015)*, with permission from Nature Publishing Group.*

The latest adult dental health survey was done in 2009 (Pitts & Chadwick, 2009). Again, there is a decrease over the years with only 6 percent of the population being edentulous compared to 22 percent in 1978. The mean number of teeth in dentate adults was 25.7. Interestingly only 17 percent of dentate adults had healthy periodontal status and 10 percent had excellent oral health. For those under 45, there is now a high chance that they will retain a large number of their permanent teeth for their whole life. This may lead to a higher percentage of decayed teeth as these are retained for longer. About one third of dentate adults had obvious decay, and for those who had decay, the affected teeth were 2.7 on average. The prevalence of decay has fallen from 46 per cent in 1998 to 28 percent in 2009 (Pitts & Chadwick, 2009).

Data from the World Health Organization (WHO) in 2000 compare mean Decayed, Missing, Filled Teeth (DMFT) in Europe for 12 years old children. England is amongst the lowest prevalence of DMFT in Europe at 0.7 along with Germany, Denmark and Cyprus. On the other hand Lithuania, Poland and Romania are among those with the highest prevalence of DMFT in Europe with 3.7, 3.2 and 2.8 respectively (Reena, 2012).

1.1.3 Aetiology

There are three theories that attempt to explain the aetiology of dental caries: the specific plaque hypothesis, the non-specific plaque hypothesis and the ecological plaque hypothesis (Takahashi & Nyvad, 2011). According to the specific plaque hypothesis, only a few different bacteria, such as *Streptococcus mutans* and *Lactobacillus spp*., are active during the disease process. In the non-specific theory, dental caries is the outcome of the activity of many species within the plaque microflora. The ecological plaque hypothesis suggests that caries is a process where demineralisation and remineralisation exist in an equilibrium and a shift in the balance will result in dental caries (Aas et al., 2008). In this process bacteria other than *S. mutans* play an important role in maintaining ecological stability. This process can be described as 'net mineral gain' and 'net mineral loss'. Once an acidic environment has been established, aciduric (acid tolerant) bacteria tend to increase and promote an environment where a 'net mineral loss' happens (Takahashi & Nyvad, 2011). Microbes produce acids upon hydrolysis of carbohydrates present in the oral cavity. Acids released by bacteria drop the pH below 5.5 and cause demineralisation of tooth tissue. Remineralisation induced by precipitation of phosphate, calcium and fluoride which are present in saliva, toothpaste, mouthwashes counters the above. Erosion of the enamel is followed by infection and demineralisation of the dentine. Dental caries will eventually reach the pulp chamber where it will cause inflammation and subsequent necrosis of the pulp tissue (Loesche, 1996, Takahashi & Nyvad, 2011).

1.1.4 Pathogenesis

Culture-based studies from plaque samples have been able to identify specific bacteria that are associated with dental caries (Becker et al., 2002, Chhour et al., 2005, Munson et al., 2004). These studies have helped with developing the above three hypotheses. *S. mutans* and *Streptococcus sobrinus* as well as *Lactobacillus* spp. have been previously identified as major bacterial agents. In dental caries *S. sobrinus* is more aciduric and acidogenic than *S. mutans*, meaning it can survive and grow in an acidic environment and produce acids respectively. However, it is in most cases outnumbered by *S. mutans* in an active carious lesion. The reason may be that *S. sobrinus* is unable to catabolize N-acetylglucosamine, a sugar present in the plaque biofilm, which in turn doesn't allow the organism to proliferate (Beighton, 2005). Only in rare cases where carbohydrate levels may be very high, for example in bulimic patients, can *S. sobrinus* proliferate in caries (Beighton, 2005). Additionally, the presence of *S. sobrinus* has been associated with high caries experience. Studies have shown that children that carried both *S.*

sobrinus and *S. mutans* had higher caries rate that children carrying only *S. mutans* (Okada et al., 2005).

In a study done in 2008 bacterial growth on teeth was analysed using a reverse capture DNA hybridization assay (Aas et al., 2008). This makes use of the polymerase chain reaction technique to rapidly detect and enumerate bacterial species. Using this technique is more sensitive than bacterial culture techniques, however it can sometimes suffer from inadequate specificity (Paster et al., 1998). Fifteen young children with primary teeth and twenty five adults were investigated in this study as well as forty controls. Plaque was removed from teeth from 4 different areas, intact enamel, white spot lesions, dentin lesions, and deep dentin lesions. The analysis presented 197 different bacterial species present in plaque. *S. mutans* was present in 90% of cases in dentin lesions. Caries also developed in the absence of *S. mutans* and individuals with high levels of *S. mutans* don't necessarily develop caries. *S. mutans* has a more dominant role in dentine lesions in primary teeth compared to permanent teeth. In early carious lesions, *Streptococcus parasanguinis* and *Streptococcus salivarius* were observed in high levels. On top of that *Corynebacterium spp.* and *Actinomyces gerencseriae* were detected in primary teeth and *Leptotrichia* spp, *Campylobacter gracilis* and *Selenomoas* spp*.* in permanent dentition. This microbial composition was completely different in deep carious lesions. The microflora was dominated *by S. mutans, Lactobacillus spp., Propionibacterium spp*. In primary teeth deep lesions, *Bifidobacterium spp.* and in permanent teeth *Atopobium Genomospecies* C1 were also identified (Aas et al., 2008). Other studies have also confirmed that *S. mutans* and *Lactobacillus* spp. are the most dominant species in deep carious cavities (Chhour et al., 2005; Fragkou et al., 2016; Corby et al., 2005).

Based on the ecological hypothesis, previous studies have shown that in a newly cleaned tooth surface *S. mutans* will only constitute 2% of the total streptococcal population (Nyvad & Kilian, 1990). In cavitated lesions, *S. mutans* will comprise 30% of the total microbial flora. In the actual advancing front *S. mutans* is seen less frequently and there, lactobacilli*,* Prevotellae and Bifidobacterium are encountered more often (Takahashi & Nyvad, 2011).

Looking more closely at the ecological hypothesis during the dynamic stability change, small changes to the pH by occasional sugary foods can be restored through homeostatic mechanisms in the saliva. As stated before, in this stage, *Actinomyces* and non-mutans streptococci are mostly present in plaque. When sugar is supplied more frequently the pH in plaque decreases. In this sugar-rich environment non mutans

streptococci increase their acidogenicity (acid production) and 'low-pH' aciduric non-mutans bacteria will increase selectively. This can lead to initiation or progression of dental caries. Upon exposure to a pH of 4.0 or less, non-mutans streptococci and *Actinomyces* lose their viability, while *S. mutans* and Lactobacilli are able to survive. This leads to the next stage where aciduric (acid resistant) bacteria are dominant. Under these condition the latter proliferate and lead to a pronounced net mineral loss and lesion progression. Similar to *S. mutans*, *Bifidobacterium* is also very acidogenic and aciduric, and this bacterium can also overcome the competition under the aciduric stage and increase its population (Takahashi & Nyvad, 2011).

It is also important to mention the role of *S. mutans* in Early Childhood Caries (ECC). A systematic review in 2006 concluded that the presence of *S. mutans*, both in plaque and saliva of young children without caries can be associated with a considerable increase in caries risk (Thenisch, et al. 2006). Similarly, the detection of *Lactobacillus* spp. and *Bifidobacterium* spp. in ECC is very common and agrees with the ecological hypothesis as these bacteria are very aciduric and acidogenic (Aas *et al.*, 2008).

The yeast, *Candida albicans* has high cariogenic potential and previous studies have shown that there is an association between ECC and *C. albicans* (de Carvalho et al., 2006; Fragkou et al., 2016).

Finally, in the table below a list of the man bacteria implicated in dental caries has been made to summarise the above review.

	Early	Deep
Primary teeth	*S. mutans* *S.salivarius* *S. parasanguinis* *Corynebacterium* spp. *Actinomyces gerencseriae*	*Bifidobacterium* spp. *S. mutans* *S. sobrinus* *Lactobacillus* spp. *Propionibacterium* spp.
Permanent teeth	*S. mutans* *S. salivarius* *S. parasanguinis* *Leptotrichia* spp. *Campylobacter gracilis* *Selenomoas* spp. *Actinomyces* spp.	*S. mutans* *S. sobrinus* *Lactobacillus* spp. *Propionibacterium* spp. *Atopobium* spp.

Table 1-1: Bacteria involved in dental caries as discussed in chapter 1.1.4

1.1.5 Streptococcus mutans

Streptococcus mutans is a facultatively anaerobic, Gram-positive coccus mainly found in the oral cavity. It is closely related to *Streptococcus sobrinus*, with which it cohabits the oral cavity. Its main virulence factors associated with cariogenicity include adhesion, acidogenicity and acid tolerance. It was first isolated and described by JK Clarke in 1924 (Clarke, 1924).

Biofilms are three dimensional structures in which the bacteria are embedded in an exopolysaccharide matrix on surfaces such as enamel. The extracellular polysaccharides (EPS), as well as other polymers such as DNA and proteins, also affect the physical and biochemical properties of the biofilm. The biofilm starts with formation of the salivary pellicle. This is formed from salivary components (such as mucin, lysozyme, proteins and other) adsorbing to the acquired enamel pellicle (AEP). AEP is the base for biofilm formation. *Streptococcus* species can adhere to the pellicle together with hundreds of other bacterial species. These species are able to ferment carbohydrates and produce acid as a by-product (Wan *et al.*, 2003). Bacteria such as *S. mutans* adhere to the pellicle via two mechanisms; sucrose-dependent and sucrose-independent. The sucrose independent path may initiate the process of adhesion but colonisation of tooth surface is mediated by sucrose dependent pathway. In the sucrose independent mechanism an interaction between particles of *S. mutans* and the acquired enamel pellicle is seen. Agglutinins found in the saliva aid the adhesion of *S. mutans*, based on the interaction with the I/II antigen, which is a multifunctional P1 adhesin anchored in the bacterial cell wall. Ag I/II interact with glycoprotein-340 which is found in saliva and adsorbs on the surface of teeth. Experiments have been done in rats to test whether there will be less adhesion if the P1 is removed genetically in *S. mutans*. Results from multiple studies arrive at mixed conclusions (Bowen et al., 1991; Crowley et al., 1999).The sucrose dependant mechanism is understood to be through the action of glucosyltransferases (GTFs) in the synthesis of glucans. GTFs are able to split sucrose into glucose and fructose. GTFs are also responsible for formation of glucans from sucrose. Glucans enable bacterial adhesion to enamel as well as bacteria to each other. After this has happened microcolonies are formed and this enables the formation of a biofilm (Krzyściak et al., 2014; Banas, 2004).

S. mutans contains a glycolytic pathway through which it can produce acids such as lactate and formate as fermentation by-products. When glucose is abundant lactate is the major fermentation product. It is understood that the acidogenicity of *S. mutans* leads to the ecological changes in the plaque flora and, in caries involving *S. mutans,* is consistent with the ecological hypothesis described above. Together with the

acidogenicity *S. mutans* is aciduric or acid-tolerant. It retains its glycolytic abilities at pH as low as 4.4 (Bender GR, 1985). Also the formation of a biofilm allows *S. mutans* to survive, whereas planktonically grown bacteria may not survive such low pH (Banas, 2004).

1.1.6 Management

The dramatic reduction in the DMFT in the past 20 years is evidence that prevention works. Prevention is based on four pillars, tooth brushing and plaque control, diet advice, fluoride and fissure sealants.

It is evident that diet advice, although very regularly given may not be as effective as once thought, and is currently thought to be only valuable for children under 5 years old (Kay et al., 2016). An example of effective diet advice may be the stopping of bottle feeding/breast feeding at night after the primary teeth have erupted. Similarly tooth brushing instructions can produce short term gains in plaque control.

In 2007 the Department of Health released a document called 'Delivering better oral health: an evidence based toolkit for prevention'. This incorporates the 4 pillars of prevention as well as advice for adults such as smoking and alcohol cessation advice (Public Health England, 2014). Importantly for paediatric dentistry it gives recommendations for the application of fluoride varnish, instructions for effective tooth brushing and correct concentrations of sodium fluoride in the toothpaste to be used. Research suggests that toothpaste containing at least 1000 µg/ml fluoride has a 23% increased preventive effect against caries compared to placebo, whereas a toothpaste with 500 µg/ml fluoride showed no difference in caries prevention compared to the placebo (Walsh et al., 2010). Fluoride varnish also has a significant effect on caries development and has been shown that applying varnish twice yearly may reduce caries by 37% in primary teeth and 43% in permanent teeth (Marinho et al., 2013). Fissure sealants are a preventive intervention which is particularly important for first molars which erupt at the age of 6-7 and are susceptible to occlusal caries. Applying a resin based fissure sealant to these teeth will reduce the chances of caries developing by 33-51% which is significant (Ahovuo-Saloranta et al., 2013). When caries has progressed, it is dealt with by surgical interventions.

Traditionally dental caries has been managed with complete caries removal and restoration of the cavity with amalgam or similar restoration. This is a very effective way of dealing with dental caries and is well evidenced (Ricketts et al., 2013). Complete caries

removal however may be demanding for young children as it will involve the use of local anaesthesia and high speed handpieces. In more recent years the focus has been on prevention as well as partial or no caries removal. With the development of new dental materials, mainly dental composites with or without antibacterial properties as well as improvements in bonding have led to less need for full caries removal and the concept of 'sealing' caries in is now well evidenced (Tell*ez* et al., 2013). One of the strongest advocates for no caries removal is the 'Hall crown' technique which led to a large amount of interest when the results of the 5 years randomized controlled trial in 2009 by N. Innes showed that sealing caries in the tooth with a preformed metal crown significantly outperformed conventional restorations (Innes et al., 2011). Since then partial caries removal or no caries removal has become very popular and sealing caries is often preferred to removing it. This is of course why dental composites with antibacterial and remineralizing properties have become the centre of interest. When caries is left behind it is vital that the marginal seal is effective to prevent caries progression. Importantly it should be mentioned that on a similar principle, non cavitated carious lesions (NCCl) can be simply sealed with a fissure sealant (Tellez et al., 2013).

1.2 Dental Materials

1.2.1 Amalgam

Amalgam has been used for over 150 years. An amalgam is formed when mercury is mixed with an alloy. The alloy used consists of a mixture of silver, tin, copper and zinc. Silver is the main component and together with tin they form Ag_3Sn, known also as the γ- phase. The γ phase reacts with mercury to form amalgam. Copper is added to increase strength. In terms of properties, amalgam has very high strength, it flows and creeps and it corrodes. Corrosion can affect the restoration in a positive and negative way; the corrosion products help to produce a good marginal seal, however it can also cause a deterioration of the properties of amalgam. The main limitations of amalgam include poor aesthetics, lack of adhesion and concerns of amalgam toxicity. The most serious potential hazard is that of mercury vapour, during placement and removal of the restoration. Dental staff are more at risk from contamination and the threshold is 50 $\mu g/m^3$. Also there have been reports of delayed hypersensitivity reactions to mercury in patients following placement of the restoration (Noort, 2007).

In January 2013 the Minamata convention was published (Mackey et al., 2014). This is an international treaty designed to protect human health and the environment from emissions and releases of mercury and mercury compounds. Dental amalgam was part

of the discussion as it accounts of approximately 3% of all mercury use in the world. The recommendation for dental amalgam is that a reduction in its use, rather than a ban, should be implemented and a greater focus on dental prevention and health promotion, increased research and development of alternatives, and best management techniques for amalgam waste should be encouraged (Mackey, Contreras and Liang, 2014). European legislation has aligned with the Minamata convention which in summary will prohibit the use of dental amalgam in under 15 years old children and in pregnant or breastfeeding women by 2018 and phase out dental amalgam by 2030 (Bourguignon, 2017).

1.2.2 Glass Ionomer Cement

Glass Ionomer Cements (GICs) are restorative materials consisting of a powder and liquid, which when mixed together produce a fluid mass which quickly becomes solid. They are mainly used in dentistry as temporary restorations, class V restorations, as luting cements and also as permanent fillings in the primary dentition. They were first described in 1971 by Wilson and Kent and they were an extension of zinc polycarboxylate cements that were available since the 1960s. The replacement of phosphoric acid with polyacrylic acid meant less irritation to the tissues and stronger restoration (Wilson & Kent, 2007). The two main advantages of GICs are the ability to bond to enamel and dentine and the ability to release fluoride over time. A further advance in GICs is the addition of a resin component, allowing the material to set by light activation. This is known as resin-modified GIC (RMGIC) (Noort, 2007). GICs are a mixture of glass, a polyacid and water. The glass comprises silica (SiO_2) and alumina (Al_2O_3) mixed with calcium fluoride (CaF_2). The polyacid can be made from copolymers of acrylic and itaconic acid or acrylic and maleic acid. Also a copolymer of vinyl, phosphoric acid is a stronger acid that has been used in more recent GICs, and gives better strength and moisture resistance. RMGICs can include polyacrylic acid, tartaric acid, a photo initiator and a hydrophilic monomer such as Hydroxyethyl methacrylate (HEMA) (Noort, 2007).

The setting reaction is with an acid-base reaction:

$MO{\cdot}SiO_2$	+	H_2A	→	MA	+	SiO_2	+	H_20
Glass		Acid		Salt		Silica gel		

Table 1-2: Setting reaction of glass ionomer cement (Noort, 2007)

There are three phases in the setting process of glass ionomer cement; dissolution, gelation and hardening. In dissolution, when the water is mixed with the powder, the acid goes into solution and reacts with the outer layer of the glass. This layer then becomes exhausted of aluminium, calcium, sodium and fluoride ions and only the silica gel is left. The hydrogen ions are released from the carboxyl groups on the polyacid diffuse on the glass and make up for the lost ions above. Although the majority of the setting reaction takes only a few minutes the GIC will continue to set for up to one month. The gelation phase happens when the calcium ions react with the carboxyl groups of the acid. Finally, the hardening phase can last up to seven days. This provides the strength to the GIC by up taking of aluminium and the introduction of crosslinks.

In the case of RMGIC the acid base reaction is similar, but much slower, enabling a longer working time. These materials may instead set rapidly upon light activation. This causes polymerization of the monomer. After 150s of the application of light source, polymerisation is almost complete (Noort, 2007; Young, 2004).

1.2.3 Biodentine

Biodentine is a calcium silicate based material which was made available by Septodont in 2011. It is important to mention it here as it is a biocompatible material with good mechanical properties. Septodont claimed that it can be used as a ‘’dentine replacement material whenever original dentine is damaged’’. It is used for crown and root dentin repair treatment, repair of perforations and resorptions, apexification and root end filling. More importantly for our research it can be used as a permanent dentine substitute in large carious lesions (Malkondu Ö et al., 2014).

Biodentine induces mineralization after its application. It increases TGF-β1 secretion from pulp cells which increases mineralization. As part of the setting process calcium hydroxide is released and due to its high pH this causes irritation, hence it induces deposition of reactionary and reparative dentine. Finally its high alkalinity causes some inhibition of microorganisms (Laurent et al., 2012).

1.2.4 Compomers

Compomers are similar to composites but they also contain a monomer/polymer with an acidic chemical group and fluoride releasing silicate glasses. They generally consist of hydrophobic monomers such as urethane dimethacrylate (UDMA) and triethyleneglycol dimethacrylate (TEGMDA) with fluoroalumino silicate glass particles. Once in contact with water an acid base reaction occurs as the compomer absorbs water, which facilities fluoride release (Meyer et al., 1998).

Polymerization shrinkage is a concern in compomers, however water sorption may compensate for the shrinkage. The water sorption doesn't initiate the glass/acid reaction significantly enough, which is a requirement to promote fluoride release (Young, 2004).

Compomers have been used as a substitute for glass ionomer cements especially in paediatric dentistry. A study has shown superior longevity compared to glass ionomers in primary teeth (Welbury et al., 2000). However the reduction in mechanical properties, especially strength of up to 40% compared to composites as well as increased polymerisation shrinkage did not allow these materials to become popular (Nicholson, 2006).

1.2.5 Composite

Dental composite consists, as the name implies, of a mixture of two or more materials. They generally consist of three different components, the organic resin matrix, the inorganic filler and the coupling agent. As we will see later however, more components may be added such as antibacterial agents and remineralising agents. The resin forms the matrix of the composite and binds to the filler particles through the coupling agent.

Composite materials are limited by polymerization shrinkage, limited toughness, the presence of unreacted monomer and other factors. Eventually these drawbacks reduce the restoration's lifetime. In recent years dental composites have been the centre of interest for researchers and dentists with a goal of improving performance.

The resin is initially a fluid monomer and is then converted into solid through a polymerisation reaction. The most common used monomer is produced from bisphenol-A and glycidylmethacrlylate (Bis-GMA). The other monomers that is common is Urethane Dimethacrylate resin (UDMA). Bis-GMA and UDMA are highly viscous fluids because of their high molecular weights. To overcome this drawback, low viscosity monomers are also available such as triethylene glycol dimethacrylate (TEGDMA). To have a long shelf-life, inhibitors, for example camphorquinone (CQ), is included to initiate polymerization.

Currently methacrylate resin formulations are included in the majority of dental composites. These are typically BisGMA and UDMA as well as BisEMA (ethoxylated bisphenol-A dimethacrylate). However, while many dimethacrylate derivatives have shown some promise, improvements relative to BisGMA/UDMA are generally modest (Noort, 2007).The filler has traditionally been Quartz. Fillers reduce polymerization shrinkage, reduce the coefficient of thermal expansion, improve mechanical properties, provide radiopacity and control colour and translucency. Finally the coupling agent ensures that the resin and the filler are bonded together strongly (Noort, 2007).

A recognised drawback of composites is polymerization shrinkage. As traditional composites do not have any intrinsic defence mechanisms against caries, once a gap is formed then microleakage will occur. The ideal composite should have as low as possible shrinkage so that it can enhance marginal adaptation, reduce chances of bond breakdown and inhibit development of recurrent caries. Typical polymerization shrinkage values range between 1.5-2.5% for conventional composites and 4-5% for flowable composites. To overcome this problem, techniques such as incremental placement or using small amounts of flowable composite in the base of boxes have been introduced (Noort, 2007). The setting reaction involves light initiated photopolymerisation of the monomers to form a highly crosslinked polymer. The polymerisation involves three separate phases, initiation, propagation and termination (Cramer et al., 2011).

1.2.5.1 Types of composites

Dental composites are generally categorized by the particle size of the filler. Traditional composites contain glass filler particles with a mean size of 10-20 μm with the largest particles reaching 40 μm. These composites had poor aesthetics and finish. Microfilled resins were introduced in the late 1970s. The particle size of 0.02 μm meant that these composites could be polished to a very smooth surface. However having such small particles means that a high resin concentration is required to cover all the surface area of the small particles. Hybrid composites overcome this by containing large particles of 15-20 μm and also a small amount of colloidal silica with a particle size of 0.01-0.05 μm. Most recent dental composites are hybrid. Finally hybrid composites with even smaller particles (average less than 1 μm) have also been developed and provide excellent aesthetics due to the high polishability (Noort, 2007). However it should also be accepted that, due to poor mechanical properties, these materials should not be used in areas of stress such as large restorations in contact with opposing tooth or to replace cusps (Fujishima et al., 1995).

1.2.5.2 Physical properties

Dental composites restorations have always been technique sensitive, require more time to place than amalgam and also require acid etching with phosphoric acid and use of a dentine bonding agent. The ideal viscosity of a composite should be a balance between how well it can be condensed in large cavities but also flow in inaccessible spaces. By reducing the filler loading a flowable composite can be produced. A more packable composite can be produced for posterior restorations by increasing filler loading. However increased filler may lead to inferior surface finish (Noort, 2007).

Biocompatibility is very important in any dental material. Dental composites release products after they set. These may include UV stabilizers, initiators and more. There have been reports of changes in oestrogen-sensitive organs and cells, mainly caused by Bis-GMA. Also allergic reactions are not uncommon. Resin based materials account for 12% of reactions to materials according to the national survey of adverse reactions to dental materials (Moharamzadeh et al., 2009).

The coefficient of thermal expansion needs to be close to that of the tooth tissue to minimize the possibility of stress developed in the tooth-composite interface when temperature changes.

Finally the aesthetic qualities of dental composite have always been well recognised. Marginal discoloration is an issue when debris penetrates in the composite-tooth interface. A good marginal seal may overcome this. Composites with large filler particles may get surface discoloration due to the increased surface roughness.

1.2.5.3 Mechanical properties

Compressive strength is easy to measure but difficult to interpret as it is a poor indicator of a materials resistance to failure (Noort, 2007). Tensile strength is a more accurate predictor of whether a material may fail, however it is a lot more difficult to measure as it is almost impossible to eliminate internal flaws or small cracks in the surface. However the diametral tensile test is an alternative which gives reproducible results and is relatively easy to measure. Hence it is often quoted in measurements of mechanical properties (Noort, 2007).

Finally wear is the process of which material is displaced by the forces generated as two surfaces rub together. A high filler loading, smooth surface finish and a strong bond between filler and resin are desirable properties on a posterior composite. Again it is

difficult to measure wear on a composite and most in vitro methods do not predict in vivo results with certainty (Noort, 2007).

1.2.5.4 Failure of composites

Dental restorations have a limited lifespan. Restorations fail due to fracture or secondary caries. Dental composites have an annual rate of failure of 3-8% and up to 70% of dental composites fail due to secondary caries or recurrent infection (Kopperud et al., 2012; Laske et al., 2016). One of the main reasons of failure is polymerisation shrinkage. As stated above dental composites may exhibit 1.5-5% shrinkage after polymerisation (Noort, 2007). This shrinkage leads to voids in the tooth tissue - composite interface which may lead to bacterial microleakage. Voids can also be created by errors in the placement of the composite such as inadequate packing (Nedeljkovic et al., 2015; Demarco et al., 2012). Finally natural degradation over time may occur due to breakdown of the resin matrix and or the interface between the filler and resin matrix (Drummond, 2008).

The table below gives a summary of the main properties of commonly used dental materials.

Property	Amalgam	(RM)GIC	Compomer	Composite
Strength	Excellent	Poor	Satisfactory	Good
Aesthetics	Poor	Satisfactory	Good	Excellent
Longevity	Excellent	Poor	Satisfactory	Good
Anti-bacterial	Yes	No	No	No
Remineralisation	No	Yes	Yes	No

Table 1-3: Properties of dental materials

1.3 Antibacterials in dental composites

1.3.1 History

Dental composites have been used in dentistry for many years to restore decayed teeth. They offer excellent aesthetics and strength. Dental composites last for an average of 7 years with the main causes of failure being fracture or secondary caries (Zhang *et al.*, 2014). Secondary caries is caused by the ingress of bacteria into the gaps between the composite and tooth. Dental composites are 3.5 times more likely to fail than amalgam restorations due to secondary caries (Bernardo et al., 2007).

Incorporating antibacterial compounds into a dental composite may reduce the risk of failure due to secondary caries. Therefore, composites with antibacterial agents incorporated that can kill bacteria in a biofilm are highly desirable. The antibacterial effect should preferably be long term and the addition of the agent should not affect the mechanical properties (Leung et al., 2005).

The antibacterial properties of dental materials have been investigated from as early as the nineteenth century where it was noted that bacteria such as *Staphylococcus aureus* and *Lactobacillus acidophilus* were killed when placed in water in contact with copper (Shay et al., 1956; Sheppard, 1935). The antibacterial properties of dental materials were first measured by Shay et al in 1951 and 1956 (Shay et al., 1956).

1.3.2 Fluoride

Fluoride works by inhibiting demineralisation and promoting remineralisation of tooth structure by forming fluoroapatite crystals. There is also an antibacterial role, however that may not be as important as the concentrations required to have an antibacterial effect are high (ten Cate & van Loveren, 1999).

Traditionally conventional glass ionomer cements have incorporated fluoride and are very effective at reducing secondary caries formation around restorations. However, compared to dental composites they are weaker. To overcome this, hybrid materials have been developed such as resin-modified glass ionomer cements and compomers. The advantage of GICs and compomers is their ability to release, absorb and re-release fluoride (Syafiuddin et al., 1997).

There are different ways to develop fluoride releasing composites. These include addition of inorganic or organic water soluble salts, addition to glass fillers and bonding to a resin component (Dionysopoulos et al., 2013).

It has also been reported that fluoride releasing filer systems, such as strontium fluoride (SrF2) and ytterbium trifluoride (YbF3) have an antibacterial effect (Beyth et al., 2014). Fluoride is released by an exchange reaction of water diffusion into the composite and fluoride release from the particles. On the downside however most of the fluoride is released by the time the setting reaction is complete and the release of fluoride in the oral cavity actually creates voids in the matrix (Beyth et al., 2014).

Antibacterial monomers containing quaternary ammonium fluoride salts are biocompatible and antibacterial against *S. mutans*. Fluoride releasing composite containing 3% of the above can inhibit bacterial growth and at the same time maintain mechanical properties similar to the control (Xu et al., 2012).

Fluoride releasing dimethacrylate monomers together with ternary zirconium fluoride chelate were investigated in 2009. The study's conclusion suggested that a composite with sustained fluoride release as well as good mechanical properties is difficult to implement, and further work is needed to improve the mechanical and physical properties (Ling et al., 2009).

In summary dental composites with sustained fluoride release achieve considerably less percentage release of fluoride when compared with glass ionomer cements (Boeckh et al., 2002; Itota et al., 2002).

1.3.3 Chlorhexidine

Chlorhexidine is a broad spectrum antibacterial agent with low cytotoxicity which has been used widely to reduce bacterial load in the oral cavity through means like mouthwash and toothpaste (Basrani, 2005).

The mechanism of action of chlorhexidine is well known. It is effective against Gram-positive and Gram-negative bacteria. The positively-charged chlorhexidine molecule is attracted to the negatively-charged sites of the bacterial cell wall. This changes the integrity of the cell membrane and chlorhexidine penetrates into the cell. This causes precipitation of the cytoplasm and prevents repair of the cell membrane. This is the bacteriostatic effect which is reversible. Greater damage to the cell membrane can be caused by increasing the concentration. Leakage of low-molecular-weight components falls and this reflects the coagulation and precipitation of the cytoplasm by formation of phosphated complexes. This stage is irreversible and bactericidal (Jones, 1997; Basrani, 2005).

In 1983 Jedryschowski et al studied chlorhexidine added to dental composite and its effect on bacteria of the oral cavity like *S. mutans* and viridans streptococci. His study showed that even the addition of 1% chlorhexidine dihydrochloride would triple the antibacterial effect compared to a conventional glass ionomer. Furthermore chlorhexidine dihydrochloride can be added to composite without affecting its mechanical properties. On the other hand chlorhexidine gluconate reduces its mechanical properties (Jedrychowski et al., 1983).

Experimental dental composites containing chlorhexidine diacetate (CHXA) have also been formulated. Chlorhexidine releasing HEMA based composites with added UDMA and TEGDMA can achieve polymerization rates suitable for clinical application. Chlorhexidine release was shown to prevent bacterial microleakage in vitro (Leung et al., 2005).

However the addition of chlorhexidine to dental composites decreases strength, increases water sorption and make the surface more porous. Simply mixing chlorhexidine with a dental composite will not meet the requirements for dental application (Deligeorgi et al., 2001).

More recently a new type of dental composite containing mesoporous silica nanoparticles (MSN), which enables recharging and sustainable release of antimicrobial agents such as CHX have been shown to be useful. The addiction of MSN to a dental composite does not affect strength, aesthetics or surface integrity of the material. In a study by Zhang et al 5%CHX and MSN addition to dental composite results in similar surface roughness and few visible voids on the surface compared to CHX alone which had rough surface and deep voids. Furthermore the CXH with MSN composite had a greater antibacterial effect than the control composite (Zhang et al., 2014).

Due to the increasing events of chlorhexidine allergy and even the death of 2 people in the UK in 2009 and 2011 from administration of chlorhexidine raises a question as to whether a known allergen should be incorporated in a dental composite (Pemberton & Gibson, 2012).

1.3.4 Polylysine

Polylysine (EPL) (Poly-ε-lysine) (ε-PL) is an antibacterial agent which is widely used due to its broad antimicrobial spectrum (Shima & Sakai, 1977). There are several types of lysine polymers which may differ in chemistry and link position. Lysine's chemical formula

is $C_6H_{14}N_2O_2$ and it has a molar mass of 146.2g/mol. It is typically produced as a homopolypeptide of approximately 25–30 L-lysine residues (Shima & Sakai,1977).

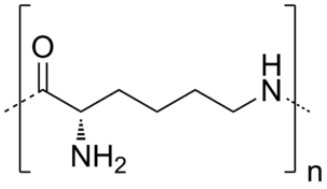

Figure 1-2: Polylysine chemical formula

Polylysine has been increasingly used as a food preservative due to its potent antimicrobial activity and safety. Its activity targets both Gram-negative and Gram-positive bacteria, yeasts and moulds (Ye et al., 2013). Polylysine is also biodegradable, water soluble, edible and non-toxic to humans. It has been approved by the US FDA (Food and Drug Administration) as safe at levels of up to 50 mg/kg in food as well as by the Ministry of Health in Japan.

Polylysine is produced for commercial and scientific purposes by *Streptomyces albulus*. It is a secondary metabolite and can be detected when the fermentation broth has an acidic pH during the stationary growth phase. Polylysine is an unusual cationic, naturally occurring homopolyamide and consists of 25 to 35 L-lysine residues with linkages between α-carboxyl groups and ε-amino groups (Yamanaka et al., 2010; Shih et al., 2006).

The mechanism against pathogens is not fully clear, however there are a few publications which confirm that polylysine has antibacterial properties (Ye et al., 2013; Shima et al., 1984). A possible mechanism could be the electrostatic adsorption of polylysine to the cell surface and abnormal distribution of the cytoplasm, which may lead to cell damage. This was suggested following the investigation of *E. coli* treated with polylysine and examined using electron microscopy. In the same article the author concluded that polylysine with more than 10 L-lysine residues have antibacterial abilities and 25-30 residues is optimum (Shima et al., 1984).

Polylysine has been used for many years. It has been given to rats in doses of 20000µg/ml with no adverse effects (Hiraki et al., 2003). Polylysine didn't cause any toxicity in reproduction, neurological and immunological functions, growth and development of embryos for two generations in rats (K. Neda, T. Sakurai, M. Stakahashi, M. Shiychi, 1999). It has a history of safe use in Japan in foods such as sushi and noodles. For example it is sprayed or dipped on the fish at levels of 1000-5000 µg/ml (Hiraki et al., 2003). It has also been used as an emulsifying agent and a dietary agent.

In medicine it has been used as an interferon inducer, drug delivery carrier and gene delivery carrier as well as coating materials for biochip and bioelectronics (Shih et al., 2006)

Polylysine has already been shown to have good antibacterial properties. In bone infections, the minimum inhibitory concentration (MIC) is 12 µg/ml against *Staphylococcus aureus* (NI Qing-Yan, LI Yan, 2008). In a different study which was done according to the National Committee for Clinical Laboratory Standards the MIC of EPL for *S. mutans* was 20 µg/ml when the initial *S. mutans* bacterial density was $5x10^5$ CFU/ml (Badaoui Najjar et al., 2009). This bacterial cell density is similar to an infected oral cavity (Pannu et al., 2013; Deo & Deshmukh, 2015).

Polylysine is considered to be a stable material under basic and acidic conditions at high temperatures. When heated to 100^{o}C or autoclaved to 121^{o}C it was stable (Hiraki, 2000).

All the above give evidence that polylysine is a safe, stable, non-toxic molecule with good antibacterial properties.

As of June 2017 there is currently one published paper investigating the use of polylysine incorporated in a dental composite and multiple doctorate thesis (Panpisut et al., 2016; Khan, 2015; Liaqat, 2015). A patent is in effect for the use of polylysine in dental composites with the inventor, Prof Anne Young, supervising this thesis.

1.3.5 Quaternary Ammonium Methacrylate and Dimethylaminohexadyl Methacrylate

In 2015 a study from Zhang et al. showed that the incorporation of a protein repellent and an antibacterial in a dental composite has a beneficial effect. Their *in vitro* study combined a protein repellent 2-methacryloyloxyethyl phosphorylcholine (MPC) with a quaternary ammonium methacrylate with increased alkyl chain length (CL) of the ammonium group. Dimethylaminohexadecyl methacrylate (DMAHDM) with CL of 16 was used as it had the best antibacterial effects. Their experiment tested 3% MPC and 1.5% against a control. These were added to BisGMA (bisphenol A glycidyl dimethacrylate) and TEGDMA (triethylene glycol dimethacrylate) (Zhang et al., 2015).

The in vitro results were promising. There was a small change in the mechanical properties, however there was a 1/10 reduction in protein adsorption and three orders of magnitude reduction in the *S. mutans* counts compared to the control composite.

In different study an antibacterial, self-healing and remineralizing composite containing DMAHDM, as well as nanoparticles of amorphous calcium phosphate (NACP) and microcapsules with poly(urea-formaldehyde) (PUF) shells, also contained triethylene glycol dimethacrylate (TEGDMA) and N,N-dihydroxyethyl-p-toluidine (DHEPT) as healing liquid. The initiator used was benzoyl peroxide. By adding up to 7.5% of self-healing microcapsules the mechanical properties of the composite were not adversely affected and self-healing was achieved with 65-81% recovery in fracture toughness, which is enough to recover the capability of a cracked composite. Additionally, this composite proved to have a strong antibacterial effect by reducing bacterial numbers in an in vitro biofilm by 3-4 logs compared to control. The inoculum used was human saliva diluted in glycerol to a concentration of 70% and then further diluted by 1:50. The two day biofilm was removed by sonicating and vortexing the discs before viable counts were done. The authors suggested that further study is needed to see if the DMAHDM has long-term antibacterial effects. Remineralizing effects were not tested in this study, although the authors concluded that the addition of NACP and DMAHDM did not affect the self-healing properties suggesting that the three components can be added to a composite without affecting its mechanical properties (Wu et al., 2015). However what is not mentioned is the cytotoxicity of some of the components of the formulations such as TEGDMA and DHEPT (Walters et al. (b), 2016; Paschalidis et al., 2014).

1.3.6 Triclosan

Triclosan (2,4,4-thrichloro-2-hydroxidiphenilethen) is an antibacterial agent which is known to be able to inhibit bacterial growth by interrupting the enzymatic activity of bacteria, specifically by inhibiting fatty acid synthesis (Kolenbrander, 2000; Wierzbicka et al., 1987; Heath et al., 1999). It is widely used in toothpastes, cosmetic products, soaps and detergents.

Triclosan has initially been used as an antibacterial agent in toothpastes, with the most common UK toothpaste with triclosan being ‘Colgate Total’. In recent years it has been introduced to dental composites. Studies previously done have had mixed results. One study showed that 1% triclosan added to a composite had a significant inhibition in bacterial growth, but another study showed that 0.3% triclosan had no antibacterial effect. It was suggested that as triclosan has limited solubility in water its release was very low (Sehgal et al., 2007; Badet & Thebaud, 2008).

1.3.7 Silver

Silver has been added to dental composites due its antibacterial effect as well as its biocompatibility (Ryu et al., 2012; Kang et al., 2009). Silver inhibits bacterial growth by interfering with bacterial enzymatic activities. Silver ions affect DNA molecules and interact with thiol groups in protein which induces the inactivation of the bacterial proteins. (Liu et al., 2013; Kawashita et al., 2000).

In one study 1% silver nanoparticles were added to a flowable composite resin. Composites discs with silver particles and a control were incubated in a suspension of *S. Mutans* and *Lactobacillus sp.* of $1x10^5$ CFU/ml for 12 hours. The suspension was then diluted and spread on agar plates. After 12 hours, there were 126 and 3.8 colonies of *S. mutans* and *Lactobacillus sp.* respectively on the control, and 0.93 and 1.2 on the composites with silver particles (Kasraei et al., 2014). In another study, the addition of 0.1 silver nanoparticles as well as amorphous calcium phosphate to a dental adhesive reduced bacterial growth by one order of magnitude after incubation for 2 days in diluted human saliva (Melo et al., 2013). A disadvantage of using silver may be the colour stability (van der Burgt & Plasschaert, 1985).

Cochrane review

The Cochrane Collaboration did a review of literature in 2013 regarding antibacterial agents in composite restorations. As always only randomized control trials were included in the review. Unfortunately, the authors concluded that at the time of writing no randomized controlled trials on resin composite containing antibacterial agent compared to a control group were available. The review also raised the important point that most of the in vitro studies to date report on bacterial load reduction rather than dental caries development (Pereira-Cenci et al., 2013).

1.4 Calcium Phosphates in Dental Composites

1.4.1 History

The term remineralisation has been used before to describe the process by which calcium and phosphate ions supplied from an external source promote ion deposition in demineralized enamel crystals to result in a net mineral gain (Cochrane et al., 2010).

Saliva alone has a great well documented remineralisation potential, due to its ability to supply calcium and phosphate ions. However, it is usually not enough to work on its own so additional extrinsic calcium, phosphate and fluoride ions are required to induce natural remineralisation (Larsen & Pearce, 2003).

Calcium phosphate is the name given to a family of minerals containing Calcium ions (Ca^{2+}) and phosphate ions (PO_4). Dental enamel consists of 90% hydroxyapatite, a calcium phosphate mineral.

Name	Abbreviation	Formula	Ca/P ratio
Hydroxyapatite	HA	$Ca_5(PO_4)_3(OH)$	1.67
Amorphous calcium phosphate	ACP	$Ca_3(PO_4)_2 \cdot nH_2O$	1.5
Tricalcium phosphate	TCP	$Ca_3(PO_4)_2$	1.5
Dicalcium phosphate	DCP	$CaHPO_4$	1
Monocalcium phosphate	MCPM	$Ca(H_2PO_4)_2$	0.5

Table 1-4: Summary of calcium phosphates discussed in this thesis

1.4.2 Hydroxyapatite

Hydroxyapatite (HA) is the second most stable and least soluble calcium phosphate after fluoroapatite. Its chemical formula is $Ca_5(PO_4)_3(OH)$. The Ca/P ratio of HA is 1.67. HA has a similar composition to bone minerals, and for that reason it has gained a lot of attention as a biomaterial component (Shi, 2004).

To prepare hydroxyapatite two types of methods are used, either solid state reactions or wet methods. The wet method consists of precipitation of calcium phosphates. In order to precipitate HA the reactant has to contain salts of calcium and phosphate (El Briak-BenAbdeslam et al., 2008). For the solid state reaction method materials are mixed to

create HA, for example calcium carbonate ($CaCO_3$) and calcium pyrophosphate ($Ca_2P_2O_7$) can be mixed at 1100^{o}C for 1 hour or potassium dihydrogenphosphate (KH_2PO_4) and calcium hydroxide $Ca(OH)_2$ at 50^{o}C for 24 hours and then at 650^{o}C for 1 hour (Rhee, 2002).

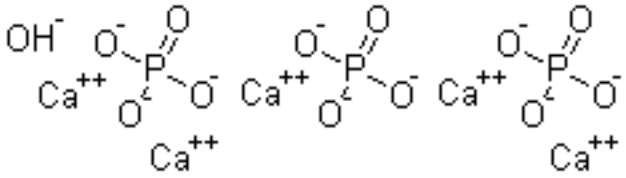

Figure 1-3: Chemical structure of hydroxyapatite

1.4.3 Amorphous Calcium Phosphate

Amorphous calcium phosphate (ACP) is a glassy precipitate of variable composition with a chemical formula of $Ca_xH_y(PO_4)_z \cdot nH_2O$ where n can be between 3 and 4.5 depending on the pH during production. The Ca/P ration can range from 0.67 to 1.50 (Habraken et al., 2016).

Unstabilised amorphous calcium phosphate can be produced as a calcium salt and a phosphate salt that could be delivered separately intraorally. This then mixes with saliva and releases calcium and phosphate ions. This unstable mixture rapidly transforms to a more stable crystalline phase, i.e. hydroxyapatite/fluorohydroxyapatite. Commercially available products with unstabilised ACP are not yet available (Cochrane et al., 2010).

Casein, which is a protein derived from milk, can stabilize calcium and phosphate ions. This has led to a remineralising technology based on casein phosphopeptide-stabilized amorphous calcium phosphate complexes (CPP-ACP). These complexes have become commercially available through chewing gums in the USA (Trident Extra Care) and dental cream (tooth mousse). Amorphous calcium phosphate (ACP) complexes are readily soluble in saliva, and create a diffusion gradient that allows them to be deposited in plaque. CPP-ACP in plaque then enters as an intact fluid and diffuses in the lesion. Formation of mineral, similar to fluoroapatite or hydroxyapatite will consume phosphate and calcium thus maintaining the gradient (Cochrane & Reynolds, 2012).

The authors of the above review concluded that there is a growing body of scientific evidence to support the use of CPP-ACP to help fluoride in inhibiting demineralization and enhancing remineralization of white spot lesions (Cochrane & Reynolds, 2012).

1.4.4 Tricalcium Phosphate

Tricalcium phosphate is a calcium salt of phosphoric acid and has the chemical formula $Ca_3(PO_4)_2$. Its Ca/P ration is 3:2. It is derived from inorganic sources such as mineral rocks. It is found in two different forms α-TCP and β-TCP. β-TCP may be prepared chemically by calcium deficient hydroxyapatite thermal decomposition at temperatures above 800^0C or by interactions of acidic calcium phosphates in a solid state phase. Also the process of calcining bones can form ion substitutes β-TCP (Dorozhkin, 2009). α-TCP may be formed by a phase transformation of β-TCP and is stable at temperatures ranging between 1125 and 1430°C. The β-TCP phase is stable up to 1125°C (Yin et al., 2003). β-TPC has been used as calcium phosphate cement, the development of multivitamins and as an ingredient in toothpastes. α-TCP is mostly used in calcium phosphate cements (Mirtchi et al., 1990).

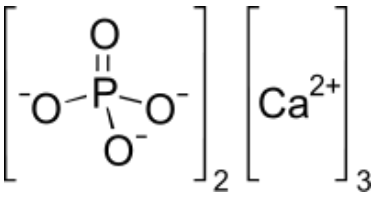

Figure 1-4: Chemical structure of tricalcium phosphate

1.4.5 Dicalcium Phosphate

Dicalcium phosphate exists in two states, dicalcium phosphate dihydrate (DCPD) also known as mineral brushite and dicalcium phosphate anhydrous (DCPA), also known as mineral monetite (Oliveira et al., 2007). DCPDs chemical formula is $CaHPO_4 \cdot H_2O$ and DCPAs formula is $CaHPO_4$. Its Ca/P ratio is 1.

DCPD is used in medicine in calcium orthophosphate cements and for tooth remineralisation (Bermudez et al., 1994). It is also added to toothpaste for caries protection when coupled with fluoride and as a polishing agent (Crall & Bjerga, 1987).

DCPA is less soluble that DCPD due to the absence of water. It is mainly used in calcium phosphate cements as well as polishing agent, toothpaste component and a source of calcium and phosphate in nutritional supplements (Takagi et al., 1998).

In dental composites dicalcium phosphate dihydrate (brushite) is not added directly to the components, instead it is formed by breakdown of monocalcium phosphate to phosphoric acid and dicalcium phosphate in contact with water, usually after the reaction with tricalcium phosphate (Mehdawi et al., 2013). As DCPA is less soluble than

monocalcium phosphate (MCP) if used in composites it will increase strength but calcium phosphate release is drastically reduced (Xu et al., 2007). This is the reason it is not used in composites, instead it is a bioproduct of the release of other calcium phosphates.

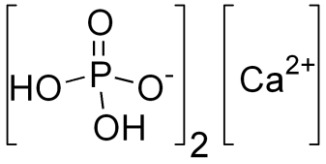

Figure 1-5: Chemical formula of Dicalcium Phosphate

1.4.6 Monocalcium Phosphate

Monocalcium phosphate is an inorganic compound commonly found as monohydrate (MCPM) or anhydrous salt (MCPA). The chemical formula of MCPM is $Ca(H_2PO_4)_2 \cdot H_2O$ and for MCPA is $Ca(H_2PO_4)_2$. Its Ca/P ratio is 1:2.

MCPM is the most acidic and most soluble CaP. Because of its high solubility it is possible to generate solutions that can kill eukaryotic cells. It is commonly used in medicine as an element in self-hardening calcium phosphate cements (Bermudez et al., 1994). It can also be used in combination with basic CaP such as TCP or CaO (calcium orthophosphate) (Huan & Chang, 2007). MCPM is added to dental composites as it is hydrophilic and upon release in solution and contact with water will form brushite and phosphoric acid. Brushite will aid remineralisation of tooth tissue and the phosphoric acid will act as etchant to enhance bonding of the material to the toot structure. MCPM has been used as buffer, hardener, leavening agent, yeast food and as a nutrient.

MCPM is produced by a reaction called ‘triple superphosphate’ for fertilizer production. Here the reaction of concentrated phosphoric acid with powdered phosphate rock produces MCPM. If the temperature is raised above 100^{o}C then a molecule of water is released and MCPA is produced (Becker, 1989).

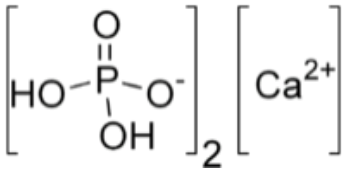

Figure 1-6: Chemical structure of Monocalcium phosphate

1.5 Monomers

1.5.1 Bulk monomers

1.5.1.1 Bis-GMA

Bisphenol A Glycidyl Methacrylate (Bis-GMA) is a high viscosity resin that has been part of dental composites for a long time. It is an aromatic ester of methacrylate. It is used as a bulk monomer on its own or together with a diluent monomer such as Triethylene glycol dimethacrylate (TEGDMA). Its formula is $C_{29}H_{36}O_8$ and the molecular weight is 512.59 (Chemfinder). Its structure includes two aromatic rings with a pendant hydroxyl group (OH). It has strong inter-molecular hydrogen bonding which arises from the hydroxyl groups and the double aromatic rings. (Asmussen & Peutzfeldt, 1998). The two pendant hydroxyl groups give Bis-GMA its hydrophilic nature. Bis-GMA has a final conversion at body temperature of 50-65% (Khatri et al., 2003).

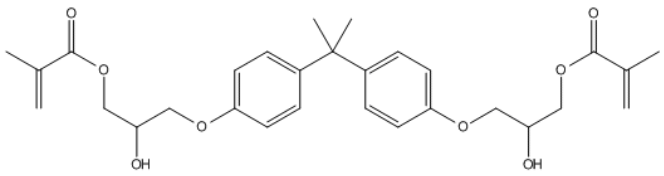

Figure 1-7: Chemical formula of Bis-GMA

Bis-GMA is the reaction product of bisphenol A (BPA) and Glycidyl Methacrylate. In recent years there has been a lot of controversy on Bisphenol-A (BPA) products. These have the ability to bind to oestrogen as well as mimic oestradiol, a hormone produced by a variety of tissues such as the ovaries and testes. While BPA is not part of any dental composite, it can be a residual impurity in composites containing Bis-GMA and it can also be released through the action of salivary esterases. However it has been shown that exposure to BPA from dental composites is minimal, at least 2000 times below the minimum daily accepted limits set by the US Environmental Protection Agency and the European Food Safety Authority (American Dental Association Council on Scientific Affairs., 2014; Kingman et al., 2012).

1.5.1.2 UDMA

Urethane dimethacrylate (UDMA) was developed by Foster and Walter in 1974. UDMA has a chemical formula of $C_{23}H_{38}N_2O_8$ with a molecular weight of 470.563. UDMA is an aliphatic high molecular weight monomer consisting of two amino groups (-NH-). These form intermolecular hydrogen bonds which increases its viscosity. However the aliphatic molecule gives it greater mobility (Filho et al., 2008). UDMA is a smaller molecule than

Bis-GMA and as a result it has a higher double bond concentration (Walters et al.(b), 2016). Composites containing UDMA instead of Bis-GMA show improve conversion, increased biaxial flexural strength and depth of cure, due to the greater flexibility (Nick J Walters et al. (b), 2016). UDMA has a significant lower viscosity and hence higher mobility than Bis-GMA. For this reason UDMA has can be used with or as an alternative to Bis-GMA in many commercial composites. Still its viscosity may be quite high, therefore more often than not a diluent monomer may be added, such as polypropylene glycol (PPGDMA) or TEGDMA. However, it can be used with a minimum amount of diluent monomer. The urethane linkage (-NHCOO-) of UDMA forms hydrogen bonds with water molecules and gives it its hydrophilic nature. These bonds are however weaker than the hydroxyl groups of Bis-GMA. This may lead to a lower hydrophilic nature than Bis-GMA (Palin et al., 2005).

UDMA has been extensively used in coatings, adhesives, pultrusion processes and dental materials. All the above are a class of materials with two methacrylate end groups, and this allows them to be cured thermally via polymerization. One of their drawbacks (common with all composites) is polymerization shrinkage which may result in marginal gaps and secondary caries (Atai et al., 2007). Dental composites containing UDMA have significantly higher monomer conversion and also better cytocompatibility compared to composites containing Bis-GMA. The use of UDMA with PPGDMA has no negative effect on the shrinkage or depth of cure and also the handling properties are better compared to Bis-GMA (Walters *et al. (b)*, 2016).

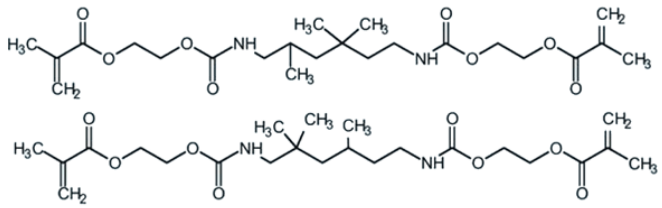

Figure 1-8: Chemical formula of UDMA

1.5.2 Diluent monomers

1.5.2.1 TEGDMA

Triethylene glycol dimethacrylate (TEGDMA) is the most commonly used diluent monomer in dentistry. It is an aliphatic hydrophilic monomer characterized by presence of ester linkages (C-O-C). It is used together with a bulk monomer such as Bis-GMA at a ratio ranging between 20-50%. It has a low molecular weight at 286g/mol and a low viscosity of 0.05 Pa·s. These can reduce the viscosity of the mixture and hence can

increase the conversion. However the increased conversion may lead to an increase in polymerization shrinkage (Floyd & Dickens, 2006; Gonçalves et al., 2009).

Concerns over TEGDMA cytocompatibility have been raised. TEGDMA has been found to be cytotoxic on pulp fibroblasts and dental pulp stem cells. Cytotoxicity can occur through apoptosis, oxidative stress or cell cycle delays (Paschalidis et al., 2014; About et al., 2002).

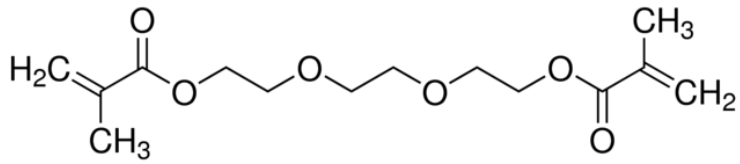

Figure 1-9: Chemical formula of TEGMDA

1.5.2.2 PPGDMA

Polypropylene glycol (PPG) is a polymer of propylene glycol which chemically is a polyether. It is used as a diluent monomer in dentistry. PPG dimethacrylate (PPGDMA) has a much higher molecular mass than TEGDMA and hence it has greater flexibility and lower double bond concentration. As a result it enables improved conversion when cured (Walters et al. (b), 2016). Furthermore it has shown reduced toxicity compared to TEGDMA (Khan et al., 2014).

An extensive search of the literature showed that PPGDMA is not a monomer commonly used in dental materials.

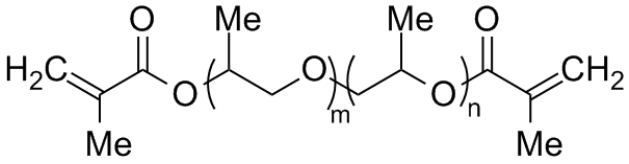

Figure 1-10: Chemical formula of PPGDMA

1.5.3 Adhesion promoting monomers

1.5.3.1 4-META

4-methacryloxyethyl trimellitic anhydride (4-META) is an acidic monomer. It has a molecular weight of 304.25 and the molecular formula is $C_{15}H_{12}O_7$. 4-META was discovered in 1978 and it marked a new level in bonding as it significantly improved adhesion to tooth structure compared to previously used adhesives (Atsuta et al., 1982;

Van Landuyt et al., 2007; Chang et al., 2002). It also has demineralizing properties (Van Landuyt et al., 2007). 4-META is a crystalline powder. After it has been added to water, a rapid hydrolysis reaction will happen and this will form 4-MET. Two carboxylic groups are produced and are attached to the aromatic group. These groups release acid and hence can cause demineralisation. The acidic and aromatic group counteract each other as one is hydrophilic and the other hydrophobic. 4-META still remains partially hydrophilic, which increases its wetting properties (Unemori et al., 2003).

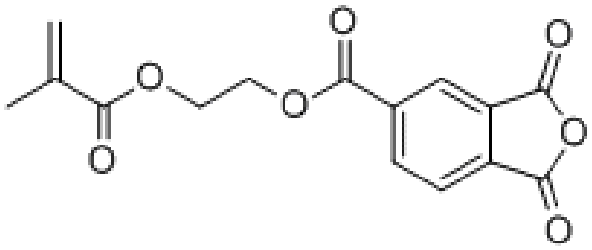

Figure 1-11: Chemical formula of 4-META

1.5.3.2 HEMA

2-Hydroxyl ethyl methacrylate (HEMA) is a monomethacrylate which is commonly used in dentistry. It is an aliphatic monomer with a molecular weight of 130.14 and a molecular formula of $C_6H_{10}O_3$. It is often added to improve the ability of the monomer to be mixed in a homogeneous solution (Van Landuyt et al., 2005). It is hydrophilic and hence it improves the wetting properties of the monomer as well as increases the penetration efficacy into the tooth structure (Nakabayashi et al., 1982; Toledano et al., 2004). The presence of hydroxyl groups (OH) allows HEMA to promote high water sorption in composites. Its hydrophilic nature allows the polymerised form to easily release components such as antibacterial agents in aqueous environments (Mehdawi et al., 2009). HEMA has also been shown to increase bond strength to tooth structure (Nakaoki et al., 2000).

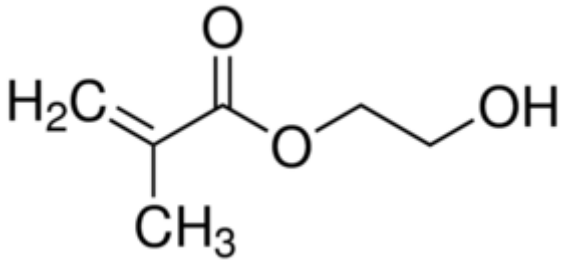

Figure 1-12: Chemical structure of HEMA

1.5.4 Initiators and Activators

1.5.4.1 DMAEMA

2-(N,N-dimethylamino)ethyl methacrylate (DMAEMA) is a co-initiator. Its chemical formula is $C_{11}H_{18}N_2O_2$. Along with N,N-dimethyl-p-toluidine (DMTP) they are the most commonly used co-initiators. They are used in conjunction with type II photoinitiators such as CQ as they have the ability to donate protons to the excited initiator molecules and form free radicals. The use of these materials, has declined due to concerns of their cytotoxicity. Materials with UDMA and PPGDMA can polymerise without the need of a co-initiator (Dunnick et al., 2014; Walters et al. (b), 2016).

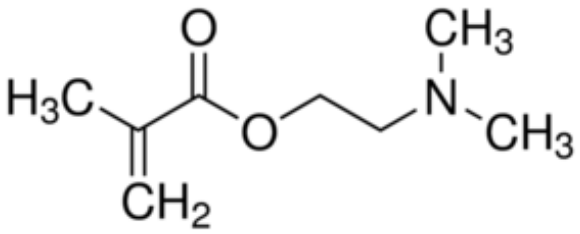

Figure 1-13: Chemical sturcture of DMAEMA

1.5.4.2 Camphorquinone

Camphorquinone (4,7,7- trimethylbicyclo[2.2.1]heptane-2,3-dione)(CQ) is an aliphatic α-diketone and is used as a photoinitiator. It has a molecular weight of 166.22 and its formula is $C_{10}H_{14}O_2$. The chemical formula is seen below. A photoinitiator is added to all composites to enable the polymerization reaction through light activation. Photopolymerization uses light to dissociate initiator molecules into free radicals. These then react with double bonds in the monomers and then crosslinking occurs. It is usually used alongside an amine for example DMAEMA, which acts as a reducing agent or as an electron and proton donor, and hence makes this an effective photoinitiating system. CQ absorbs light in the ultraviolet region at 200-300nm and in the visible region at 467nm. This is responsible for its yellow colour (Kamoun et al., 2014). Previously UV light was used to cure dental composites, however as this provided limited depth of cure, it has been replaced with high intensity visible light sources, such as light emitting diodes (LED) or halogen light (Bala et al., 2005). Absorption of light by CQ leads to two excited states, ^{1}CQ* and ^{3}CQ*. Electron transfer between the two states and an amine forms an exciplex. This generates free amine radicals, which initiate polymerization of dimethacrylates by hydrogen abstraction (Shi & Nie, 2007). Recent studies in this group have shown that amines such as DMAEMA can be cytotoxic, so they are not used anymore as CQ can also be used on its own without an amine (Walters et al. (b), 2016).

However CQ/amine complex can be more efficient than CQ alone (Taira et al., 1988). Benzoyl peroxide (BPO) is an alternative to CQ and used in chemical cured composites. Resins containing polymer and BPO and a liquid monomer are mixed releasing polymerized polymer chains. When the mixture reaches 60^{o}C, BPO undergoes homolytic decomposition of the peroxy bond. This thermolylsis releases radicals which initiate the polymerization reaction (Kwon et al., 2012).

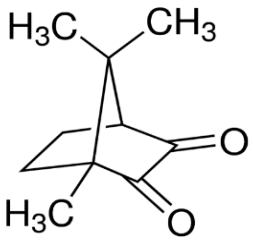

Figure 1-14: Chemical formula of CQ

1.6 Summary

Dental caries is one of the most common diseases affecting humans. Millions of pounds are spent yearly in the UK to treat it, while a large proportion of the population still has untreated dental caries.

Having dental caries can have a detrimental effect on a person. It can lead to pain, swelling, loss of teeth and difficulty eating and drinking. There is evidence showing that invasive dentistry can create anxiety and young 5 year old children who have had pain/invasive dental treatment are more likely to be dentally anxious at the age of 10 (Oosterink et al., 2008; Raadal et al. 2002). This can lead to avoidance of dentists and further problems. This avoidance can usually be associated with high caries rate (Arnrup et al., 2003).

In recent years the concept of ‘’Drill and Fill’’ has changed remarkably and dentistry is now focused in prevention, early diagnosis of caries and non-invasive treatments. Carious teeth are in many cases not fully cleaned from caries before being restored. This is mainly due to the evolution of dental materials, primarily GIC’s and RMGIC’s, which has allowed for less invasive dentistry. Such materials may be able to arrest the development of caries, remineralise dental hard tissue and inhibit the remaining bacteria that may have been left in the cavity. The lack of strength has however proved problematic. There have been multiple attempts to create composites with antibacterial as well as remineralising properties, however there are currently no published clinical

trials with any such composites. These have been tested in vitro. Novel dental composites containing antibacterial polylysine are currently under development at the Eastman Dental Institute and are the focus of this study. It is anticipated that the release of polylysine may enhance the antibacterial properties of the novel composites.

2. Chapter Two: Aims and Objectives

2.1 Hypothesis

The addition of a small amount of polylysine into a novel composite will increase the antibacterial properties of this composite when compared to control.

2.2 Aims

The aim is to produce a dental composite that has the potential to inhibit the growth of *S. mutans*.

2.3 Objectives

1. Determine the minimum inhibitory and minimum bactericidal concentration of polylysine against *S. mutans* and compare it with chlorhexidine, a known potent antibacterial agent.
2. Assess antibacterial properties of
    a. UDMA / TEGDMA based composite discs with fixed MCPM / TCP and 4 different concentrations of polylysine (EPL).
    b. Z250, UDMA and UDMA / PPGDMA based composite discs with varying concentrations of MCPM and EPL.
    c. Z250, FUJI II, FUJI IX, UDMA / PPGDMA based composites discs with varying concentrations of MCPM and EPL tested in air and air enriched with 5% CO_2 and 1% sucrose.
    d. Z250, FUJI II, UDMA / PPGDMA based composite discs with varying concentration MCPM and EPL in different inoculum concentrations.
3. Visualise bacterial growth on UDMA / PPGDMA based composite discs containing MCPM and polylysine and compare it with commercial materials.
4. Determine how different concentration of polylysine in UDMA / PPGDMA based composite discs affect the mass and volume over time.
5. Determine the amount of polylysine released from UDMA / PPGDMA based composite discs containing polylysine.

3. Chapter Three: Materials

3.1 Components

Below are tables of all components used to create the novel composite formulations that were used in this thesis.

Material	Brand	Lot/batch number	Series used
40nm fumed silica	Aerosil OX-50, Evonik Industries AG, Essen, Germany	153022145	All
0.7µm glass	DMG, Hamburg, Germany	DMG 021110 Batch: 706366	1
7.0µm glass	DMG, Hamburg, Germany	DMG 020684 Batch: 680326	1
0.7µm glass	DMG, Hamburg, Germany	DMG 021100 Batch: 746375	2, 3 and 4
7.0 µm glass	DMG, Hamburg, Germany	DMG 020684 Batch: 711591	2, 3 and 4
Polylysine	(Epoly^{tm}P) Handary, Belgium	0204	1 and 2
Polylysine	(Epoly^{tm}P) Handary, Belgium	0201	3 and 4
MCPM (53µm)	Himed, Hitemco medical applications, USA	MCP-B26	All
TCP (16.9µm)	Plasma Biotal Limited, UK	P 292 S	1
CQ	DMG, Hamburg, Germany	DMG 100134 Batch: 90339	All

Table 3-1: Powdered components

Material	Brand	Lot/batch number	Function	Molecular weight (g/mol)	Series used
UDMA	DMG, Hamburg, Germany	DMG 100112 Batch: 97406	Bulk monomer	470.6	All
TEGDMA	DMG, Hamburg, Germany	88661	Diluent monomer	286.3	1
PPGDMA	Polysciences, Inc., USA	626208	Diluent monomer	600.0	All
4-META	Polysciences, Inc., USA	595697	Adhesive monomer	304.2	All
HEMA	DMG, Hamburg, Germany	88161	Adhesive monomer	130.14	1
DMAEMA	Sigma-Aldrich, Gillingham, UK		Activator	157.21	1

Table 3-2: Liquid components

3.2 Novel composite formulations with added polylysine

Composites pastes were prepared in four different sets of formulations (see tables 5-14). The liquid phase was prepared by mixing the components and stirring for twenty-four hours at room temperature on a magnetic stirrer hot plate (Jeo Tech) until a clear liquid was achieved.

Powder and liquids were mixed for 45 seconds at 3500rpm using the centrifugal mixer (Speedmixer, Hauschild Engineering, Germany) to produce pastes. This method minimises air present in the paste and ensures full wetting of all the particles. The glass used remained constant in all formulations and was composed of 7.0μm, 0.7μm and 40nm fillers mixed at a ratio of 6:3:1. There were three different monomers prepared, two different calcium phosphates used and all pastes contained polylysine apart from the controls. Disc shaped specimens were formed by applying the composite pastes to metal circlips with internal diameter 10mm and thickness of 1mm and pressing the between two sheets of acetate. This not only prevented oxygen inhibition during polymerization but also expelled excess paste, ensuring an even distribution of the same thickness. The specimens were then photopolymerized using a blue light emitting diode curing unit with

a wavelength of 450-470nm and power of 1100-1300 mW/cm^2 (Demi Plus, Kerr Dental). The curing duration was 40 seconds on each side of the disc. A circular motion was used while curing to ensure fully polymerised disks, as the width of the curing unit beam was 7mm and the discs 10mm. The curing unit was kept approximately 5mm away from the specimen. The disks were then removed from the circlip and any excess on edges removed and polished. They were then stored at room temperature away from light until the day of the experiment.

Below a schematic of the steps for preparation of composites discs is shown.

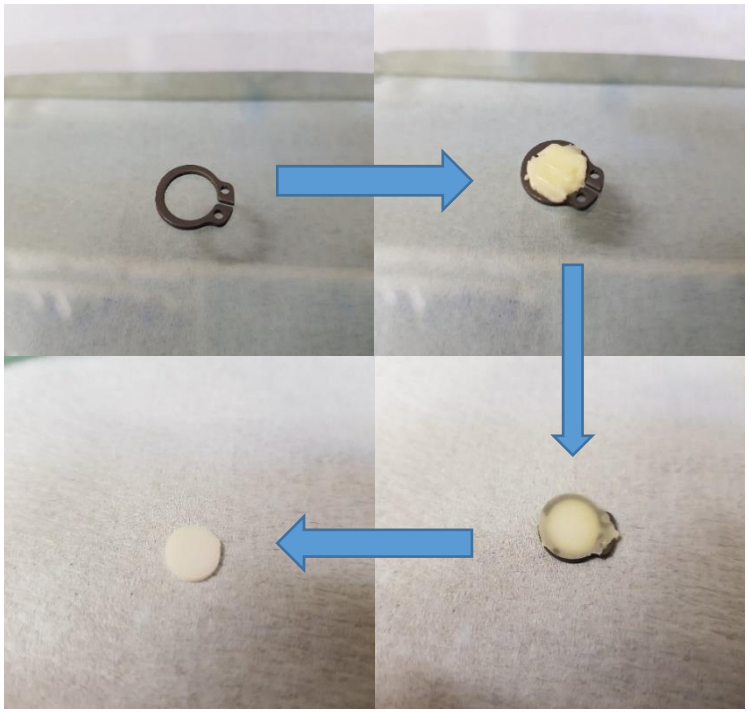

Figure 3-1: Preparation of composite disc

3.2.1 Series 1 - UDMA/TEGDMA composites with MCPM/TCP and varying EPL

The first series included 4 composites with constant monomer and glass but different concentrations of polylysine. These were 0%, 2%, 5% and 10%. The powder to liquid ratio (PLR) was 4:1 and the same monomer was used in all composites.

Below is a table showing the 4 composites that were prepared.

Composite	**Monomer**	**Glass 7µm, 0.7µm, nano (wt%)**			**MCPM (wt%)**	**TCP (wt%)**	**EPL (wt%)**
A1	UDMA/TEGDMA	51	25.5	8.5	7.5	7.5	0
A2		49.8	24.9	8.3			2
A3		48	24	8			5
A4		45	22.5	7.5			10

Table 3-3: Table of powder phase of series 1

Monomer	**UDMA:TEGDMA (wt:wt)**	**HEMA (wt%)**	**4META (wt%)**	**CQ (wt%)**	**DMAEMA (wt%)**
UDMA/TEGDMA	3:1	2	5	0.75	1

Table 3-4: Table of liquid phase of series 1

3.2.2 Series 2 - UDMA and UDMA/PPGDMA composites with varying MCPM/EPL

The second series included a combination of different composites. The glass used remained constant but was different to the 1st set as the distributor provided us with an updated batch. The powder to liquid ratio was 5.

Below is a table that shows the 5 composites that were prepared as well as the commercial composite, Z250. Z250 (3M) is a popular dental composite, the successor of Z100, first released in 1992. It consists of 2 monomers, a blend of UDMA and Bis-EMA. Both monomers have high molecular weight. The filler has a particle size of 0.01µm to 3.5µm with an average particle size of 0.6µm.

Composite	Monomer	Glass 7µm, 0.7µm, Glass nano (wt%)			MCPM (wt%)	EPL (wt%)
Z250	UDMA/ Bis-EMA	Average particle size of 0.6µm			0	0
B1 (control)	UDMA/PPG	60	30	10	0	0
B2	UDMA/PPG	52.8	26.4	8.8	10	2
B3	UDMA/PPG	55.8	27.9	9.3	5	2
B4	UDMA/PPG	54	27	9	5	5
B5	UDMA	54	27	9	5	5

Table 3-5: Powder phase of series 2

Monomer	UDMA:PPG (wt:wt)	4META (wt%)	CQ (wt%)
UDMA/PPG	3:1	3	1

Table 3-6: Liquid phase of series 2

3.2.3 Series 3 - UDMA/PPGDMA composites with varying MCPM/EPL and PLR

In the third series there were no new components introduced. Instead the powder to liquid ratios was changed to assess if it would have any effect in the antibacterial properties. Two formulations from the 2nd set of composites were used, B2 and B4 with the only difference that the PLR was changed to three. The polylysine was changed to an updated batch which was the same brand and chemical formula as with the 2nd batch of composites (Epoly™ P) (Handary, Belgium).

Two more commercial materials were also used, GC FUJI II LC and GC FUJI IX (GC America). FUJI IX is a glass ionomer restorative cement which has the ability to release fluoride and sets through an acid / base reaction as described previously. FUJI II LC is a resin modified glass ionomer restorative cement which again releases fluoride and sets initially by HEMA free radical polymerisation through light activation. This is followed by a water catalysed acid / glass reaction. They both have a PLR of about 3:1 making them quite flowable. They release fluoride as described in chapter 1.2.2. The commercial composite Z250 was also used.

Below is the table that shows the 5 composites that were prepared. Two formulations were prepared in PLR 5 and 3, whilst the control was PLR 5 only.

Composite	Monomer	Glass 7µm, 0.7µm, nano (wt%)			MCPM (wt%)	EPL (wt%)	PLR
C1 (control)	UDMA/PPG	60	30	10	0	0	5
C2		52.8	26.4	8.8	10	2	3
C3					10	2	5
C4		54	27	9	5	5	3
C5					5	5	5

Table 3-7: Powder phase of series 3

Monomer	UDMA:PPG (wt:wt)	4META (wt%)	CQ (wt%)
UDMA/PPG	3:1	3	1

Table 3-8: Liquid phase of series 3

3.2.4 Series 4 - UDMA/PPGDMA composites with varying MCPM/EPL

In the fourth series all the components remained constant apart from calcium phosphate and polylysine. This batch had a constant monomer which included UDMA and PPG for all formulations including the control. The powder to monomer ratio was 5:1, same as on the previous sets of composites.

Below is a table that shows the seven composites that were prepared. For the microscopy, mass/volume change and polylysine release these were named differently for clarity.

Composite	Also named	Monomer	Glass 7µm, 0.7µm, nano (wt%)			MCPM (wt%)	EPL (wt%)
D1 (control)	0M 0P	UDMA/PPG	60	30	10	0	0
D2	10M 0P	UDMA/PPG	54	27	9	10	0
D3	10M 0.5P	UDMA/PPG	53.7	26.9	8.9	10	0.5
D4	10M 1P	UDMA/PPG	53.4	26.7	8.9	10	1
D5	10M 2P	UDMA/PPG	52.8	26.4	8.8	10	2
D6	5M 5P	UDMA/PPG	54	27	9	5	5
D7*	0M 2P	UDMA/PPG	58.8	29.4	9.8	0	2

Table 3-9: Powder phase of series 4 (D7 only used in polylysine release)*

Monomer	UDMA:PPG (wt:wt)	4META (wt%)	CQ (wt%)
UDMA/PPG	3:1	3	1

Table 3-10: Liquid phase of series 4

3.3 Confocal microscopy

Composite formulations from series 4 were used for this experiment. The formulations used were D1-D6 and they were named according to their MCPM and EPL concentrations.

3.4 Scanning Electron Microscopy

Composite formulations from series 4 were used for this experiment. The formulations used were D1, D2, D4, D5 and D6 and they were named according to their MCPM and EPL concentrations.

3.5 Mass and Volume

Composite formulations from series 4 were used for this experiment. The formulations used were D3-D6 and they were named according to their MCPM and EPL concentrations.

3.6 Polylysine release

Composite formulations from series 4 were used for this experiment. The formulations used were D3-D7 and they were named according to their MCPM and EPL concentrations.

4. Chapter Four: Methods

4.1 Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations

The aim was to establish the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of polylysine for *S. mutans* (Objective 1). In these experiments, chlorhexidine was tested as a control since it has been tested extensively in composites before.

Although there is no Clinical Laboratory Standards method for determining MIC/MBC for *S. mutans,* we used bacterial densities from $5x10^{5-8}$ CFU/ml as these are common concentrations used in antibiotic susceptibility testing.

MIC and MBC determination

Polylysine (EPL) (Epoly^{tm}P Handary, Belgium, Batch:0201) and Chlorhexidine diacetate salt hydrate (CHX) (Sigma-Aldrich, UK batch: 083K0014V) were prepared to 1wt% (10000µg/ml) concentrations by adding 0.1 g of each to 10 ml of distilled water (DW).

S. mutans UA159 (domestic strain) was stored at -80°C in brain heart infusion broth (BHI) (CM1135 brain heart infusion, Oxoid and CM1136 brain heart infusion agar, Oxoid) containing 10% (v/v) glycerol. It was cultured in an atmosphere of air enriched with 5% CO_2 on BHI agar or in BHI broth. To prepare the inoculum for determination of polylysine MICs and MBCs, a single colony was removed from a 72 h BHI agar culture and inoculated to BHI broth which was incubated in air enriched with 5% CO_2 at 37°C for 16 hours. It was assumed that a single colony is derived from a single bacterium. The culture was diluted 1 in 3 with BHI and then the Optical Density (OD_{600}) measured to approximately 0.40-0.50 using a spectrophotometer (Biochrom WPA CO8000). This culture has a density of approximately $5x10^8$ CFU/ml. The culture was then diluted accordingly depending on the density of inoculum required. To determine accurately the inoculum density, a ten fold dilution series was prepared and plated on agar. The number of colonies that grew following three days of incubation in air enriched with 5% CO_2 were counted and used to calculate the number of viable bacteria in the inoculum after consideration of the dilution factor.

The MIC of EPL and CHX were determined using 96 well tissue culture plates as follows: These were prepared to contain 100µl of Brain heart infusion broth (BHI) and the antibacterial agent at different concentrations. Two-fold serial dilutions were then done from 5000 µg/ml to 100 µg/ml for EPL and from 100 µg/ml to 1µg/ml for CHX. The first

column was used as control. 100µl of the inoculum was then added to all wells for a total of 200µl per well. The well plate was incubated in air enriched with 5% CO_2 for 24 hours. In some occasions, different serial dilutions were done to obtain more accurate results.

After 24 hours, there was growth in the wells where the antibacterial was not enough to inhibit the bacteria and wells without growth were clear. The MIC was the lowest concentration of antibacterial that resulted in a clear well.

To set up the MBC a 96- well inoculator was sterilised with 70% ethanol and then dipped into the wells once and immediately transferred onto a 12cm agar plate until contact was made with the agar and then withdrawn. Any live bacteria will grow to form colonies and can be counted by assuming that each single colony arises from a single bacterium. The agar plate was incubated in air enriched with 5% CO_2 for three days to allow for bacterial growth. After 72 hours the results of MBC were obtained. MBC will always be higher/equal to MIC.

4.2 Novel composite formulations with added polylysine

The aim was to find how different components added to the composite formulations inhibits the growth of *S. mutans* or reduces the viable counts (Objectives 2 - a, b, c, d).

The composite formulations used can be found in the materials section.

The suspension of *S. mutans* was prepared to different concentrations with the same method as the MIC/MBC experiment.

Below the method of investigating the antibacterial properties of the novel composites is described in detail.

Sixteen hours before the experiment the BHI broth was inoculated with *S. mutans* for overnight growth. This was then diluted as required for the inoculum concentration needed as described before. On the day of each experiment the discs were irradiated with ultraviolet light for 30 minutes on each side to ensure bacterial decontamination. This was done by first placing the composite discs in a 24-well plate where the experiment would be carried out and placing the well plate in a custom-made UV box. The well plate was flipped at 30 minutes to ensure full decontamination.

The composite discs were placed into a 24 well plate as seen in figure 4-1. Then 1ml of the inoculum was added to the well plates and the plates are placed on a shaking tray at 200 rpm in air at 37^{o}C or in air enriched with 5% CO_2 non shaken at 37^{o}C.

At 6 hours, the viable bacterial counts were calculated by preparing a 10-fold dilution series of each suspension and plating 100 µl aliquots on BHI agar plates. A 100 µl volume of the undiluted suspension was also plated when the bacteria remaining were expected to be very few. In some occasions where complete kill was expected, the whole 1 ml suspension was centrifuged (Jouan C412, Fischer Scientific) for 15 minutes at 3000rpm to recover the bacteria which were resuspended in 100 µl of broth and plated in agar. The number of colonies that grew following three days of incubation of all the agar plates in air enriched with 5% CO_2 was counted and along with the dilution factor used to calculate the bacterial counts for each composite disc.

At 24 hours viable counts were repeated in the same way as above. Bacterial colonies were visible on the agar plates after 3 days of incubation in 5% CO_2.

The results are based on 3 composite discs per experiment and the experiment was repeated on 3 different occasions to further increase confidence.

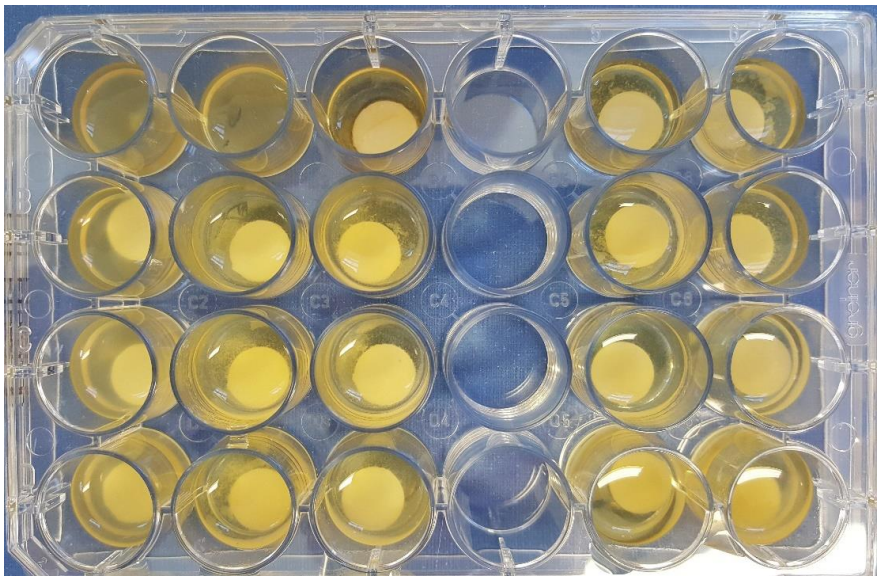

Figure 4-1: Example of 24 well plate with composite discs and 1ml of suspension of S. mutans in BHI

4.2.1 Series 1 - UDMA/TEGDMA composites with MCPM/TCP and varying EPL

The aim was to determine the minimum concentration of polylysine that will inhibit bacterial growth (objective 2a). The first series of composites was tested in air while shaken with an inoculum density of $6x10^4$ CFU/ml.

4.2.2 Series 2 - UDMA and UDMA/PPGDMA composites with varying MCPM/EPL

The aim was to see if calcium phosphate and diluent monomer would affect the antibacterial properties (objective 2b). Additionally a higher inoculum density was used, $1x10^5$ CFU/ml.

4.2.3 Series 3 - UDMA/PPGDMA composites with varying MCPM/EPL and PLR

The aim was to test the antibacterial properties of composite formulations under different conditions, air and air enriched with 5% carbon dioxide, at an inoculum density of approximately $6x10^6$ CFU/ml (objective 2c).

4.2.4 Series 4 - UDMA/PPGDMA composites with varying MCPM/EPL

The aim was to test the antibacterial properties of composite formulations under different inoculum concentrations, $8x10^5$ CFU/ml and $8x10^6$ CFU/ml (objective 2d)

4.3 Confocal microscopy

4.3.1 Technique

Confocal microscopy is an optical imaging technique, which provides increased optical resolution and contrast by adding a spatial pinhole at the confocal plane of the lens to eliminate out of focus light. It is able to collect different images of the specimen at different depths and thus able to create a three dimensional image. Its difference compared to a conventional microscope is that it can ‘see’ images at deeper levels, where a conventional microscope would not be able to provide enough light to penetrate (Smith, 2006).

4.3.2 Method

Confocal (Radiance 2100, Biorad) was used in this thesis to visualise alive and dead bacteria on the composites discs after 3 day exposure to a suspension of *S. mutans*

(objective 3). In our experiments composite discs, which were previously prepared and immersed in a suspension of *S. mutans* for 96 hours, were stained using the LIVE/DEAD Viability kit (Thermofischer Scientific, UK). The LIVE/DEAD kit contains calcein and ethidium homodimer-1 (EthD-1) in the presence of DNA. Our aim was to visualise live and dead bacteria on the discs. Live cells are identified by the presence of ubiquitous intracellular esterase activity, which is determined by the enzymatic conversion of the nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcein. Calcein produces a uniform green fluorescence in live cells. EthD-1 enters the cells that have damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, which then produces a bright red fluorescence in dead cells. Background fluorescence is very low as the dyes are virtually non-fluorescent before coming into contact with the cells.

After the discs were removed from the suspension of *S. mutans* they were gently immersed in fresh Brain Heart Infusion broth before placed in a clean well plate. The LIVE/DEAD assay was prepared by mixing 1μL of each of the two liquids with 1ml sterilized deionized water. The solution was kept out of sunlight and was stored at <-20^{o}C between experiments. Using a pipette 20μl of the assay was placed on the surface of the disc. The assay reacts very quickly so the working time is no more than two hours.

When composite discs from the viable counts experiments were used the conditions were slightly different. The discs were incubated in air or air enriched with 5% CO_2 for 3 days. Sucrose was added to the suspension of *S. mutans* at a concentration of 10mg/ml. The initial bacterial density was 5x10^6 CFU/ml.

An objective lens of 10x-20x magnification was used to visualize dead or alive colonies of *S. mutans* under the Confocal. These were processed and saved using Lasersharp 2000 application. The images saved were separate for alive and dead colonies. These were then stacked to show both alive and dead colonies together. This was done using ImageJ (ImageJ Developers).

4.4 Scanning Electron Microscopy

4.4.1 Technique

Scanning Electron Microscopy (SEM) uses a focused beam of electrons targeted on the surface of the specimen to generate the images. The electrons in the beam interact with the specimen and produce signals which can be used to obtain information on the surface topography and composition. A beam of electrons is generated by the electron

gun. This travels through an electromagnetic field and hits the sample. A set of electromagnetic coils pull the beam back and forth scanning it across the specimen's surface. The electrons which are emitted by atoms excited by the electron beam, known as secondary electrons, are gathered and converted into the signal which produces the final image on the screen. Whereas conventional microscopes which use light have a maximum magnification of about 1000x, the SEM which uses electrons can magnify specimens up to 30000x.

4.4.2 Method

SEM (Phillip XL-30, Eindhoven, The Netherlands) was used in this study for the visualisation of bacterial growth on the composite discs after exposure to a suspension of *S. mutans* (objective 3). The same discs that were used for viable counts at 24 hours were left in the suspension of S*. mutans* until 96 hours in air enriched with 5% CO_2 to allow for a biofilm to form. These were then removed from the suspension and were fixed in 3% glutaraldehyde (Agar Scientific, UK) in 0.1M sodium cacodylate buffer for a minimum of 24 hours. Before using the SEM the discs were dehydrated in a series of different concentrations of ethyl alcohol for 10 minutes each. Following that, the discs were immersed in hexamethyldisilazane (TAAB Ltd, UK) for 2 minutes and then coated with platinum in a sputter coating machine (Polaron E5000, East Sussex, UK) for 1 minute and 30 seconds at 20 mA. After that they were mounted on aluminium stubs with an adhesive. The discs were then loaded into the SEM and images were taken. Different magnifications were used to visualise bacterial growth. Individual bacteria can be seen at a magnification of approximately 2500x-5000x. A similar technique to prepare and visualise *S. mutans* has been used before (Asahi *et al.*, 2015).

4.5 Mass and Volume

Mass and volume change of different composite formulations were evaluated using a digital balance with a density kit (AG204, Mettler, Toledo) (objective 4).

Composite discs were prepared in seven different formulations. The discs were prepared in 10mm diameter by 1mm thickness. Three composites discs were used per formulation. The discs were immersed in 1ml of deionised water (DW) in a sterile tube. At different time points (1, 3, 6, 24, 48, 120, 168, 336, 672 and 1344 hours) the discs were removed from the solution, dried in absorbent paper, weighted in air and in the buoyancy medium and placed into new tubes. The medium used to measure the body volume was 1%

Sodium dodecyl sulphate in DW. The temperature of the medium was recorded at each time point as the density of the medium changes at different temperatures. The specimens were kept at room temperature in between experiments at 23 degrees Celsius. The mass (m), volume change (v) and density (ρ) were determined using the equations below and according to the ISO 17304:2013.

- The **mass** is measured in grams using the density kit.
- The **volume** can be calculated using the below formula:

$$v=\frac{m}{\rho}$$

where:

v is the volume in cubic centimeters

m is the mass in g

ρ is the density in g/ml

- The **density** of the specimens was calculated using the below formula:

$$\rho=\frac{m_{c1}\,\rho_0}{m_{c1}-m_{c2}}$$

where:

ρ = the density of the specimen in g/ml.

m_{c1}= the mass of the specimen in air and

m_{c2} = the mass of the specimen in the buoyancy medium, both measured in grams

ρ_0 = the density of the buoyancy medium at the measuring temperature in g/ml

- Mass and volume change percentage can be calculated at each time point using the below equations:

$$m\ (\%) = 100 \times \frac{m_t - m_0}{m_0}$$

$$v\ (\%) = 100 \times \frac{v_t - v_0}{v_0}$$

m_0 is the sample mass initially and v_0 is the sample volume initially.

m_t is the sample mass at a given time and v_t is the sample volume at a given time.

Means and standard deviations of the samples were calculated and plotted against the square root of time (SQRT).

4.6 Polylysine release

4.6.1 Technique

High-performance liquid chromatography (HPLC) is an analytical technique, generally used for separation, identification and quantification of different components in a mixture. HPLC is commonly used to separate and quantify hydrophilic drugs. In this thesis, HPLC was used for the determination of polylysine concentration. HPLC operates by passing pressurized liquids containing the sample through columns filled with the adsorbent material. The components interact differently and lead to their separation as they flow out of the column. Several techniques have been suggested before for the determination of polylysine release in solution with the most common one being the use of trypan blue (TB) assay (Grotzky et al., 2010). The advantage of HPLC is that it is less sensitive to release of additional components and can show differences arising through changes in polylysine molecular weight.

4.6.2 Method

HPLC was used to measure polylysine release from composite discs (objective 5). The composite discs were prepared in 10mm diameter x 1mm thickness. Three samples were prepared for each different formulation. The samples were initially weighed and then immersed in 1ml deionized water (DW). The discs were removed from the DW, weighted and placed in the next tube at certain time points. They were evaluated for three weeks. The specific time points were 1, 3, 6, 24 hours, 2, 5, 7 days and 3 weeks.

To prepare the solutions for HPLC, 300µl of the DW were transferred to the HPLC vials. These were then inserted in the HPLC.

A normal phase column in hydrophilic interaction liquid chromatography (HILIC) mode was used for the experiment. HILIC mode us a HPLC technique used to analyse polar compounds, charged substances, carbohydrates and peptides. This mode employs polar stationary phase such as silica and acetonitrile (ACN) is commonly the mobile phase (Venkatasami & Sowa, 2010). The benefits of using ACN are its miscibility in water, low absorbance at short wavelength (<280nm), high elution strength and low pressure applied to the column (Buszewski & Noga, 2012). Analysis of solutions were performed using Shimadzu HPLC system (Shimadzu corporation, Kyoto, Japan). The

mobile phase was made of 1l of acetonitrile with 1ml of added 97% phosphoric acid and 1l of DW with 1ml of added 97% phosphoric acid. The flow rate was set to 1.0 ml/minute and each sample had an average run time of 43 minutes. The experiment was run at 30^{o}C. Spectroscopic detection was used to detect polylysine in the solution through ultraviolet (UV) absorption. Absorbance was recorded at 210 nm. Polylysine chromatographic peak will be detected between 19 and 27 minutes. The values obtained by the HPLC are essentially absorbance of UV light and to measure this the surface area underneath the chromatographic peak was calculated using 'Chromeleon' software by 'Thermoscientific'.

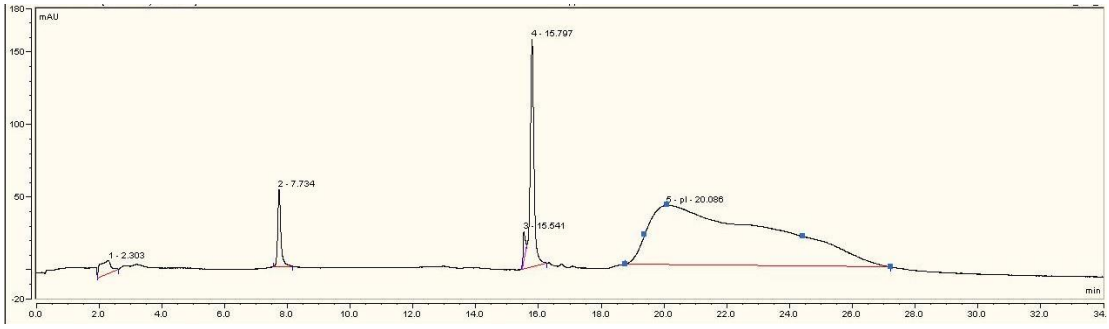

Figure 4-2: Example of chromatograph of EPL. The area below the peak at 18-26 minutes was measured.

Calibration was done by preparing a polylysine stock solution and then diluted to 100, 80, 60, 40, 20, 10 µg/ml. A calibration curve was made by plotting the Polylysine concentration (µg/ml) vs UV response (mV).

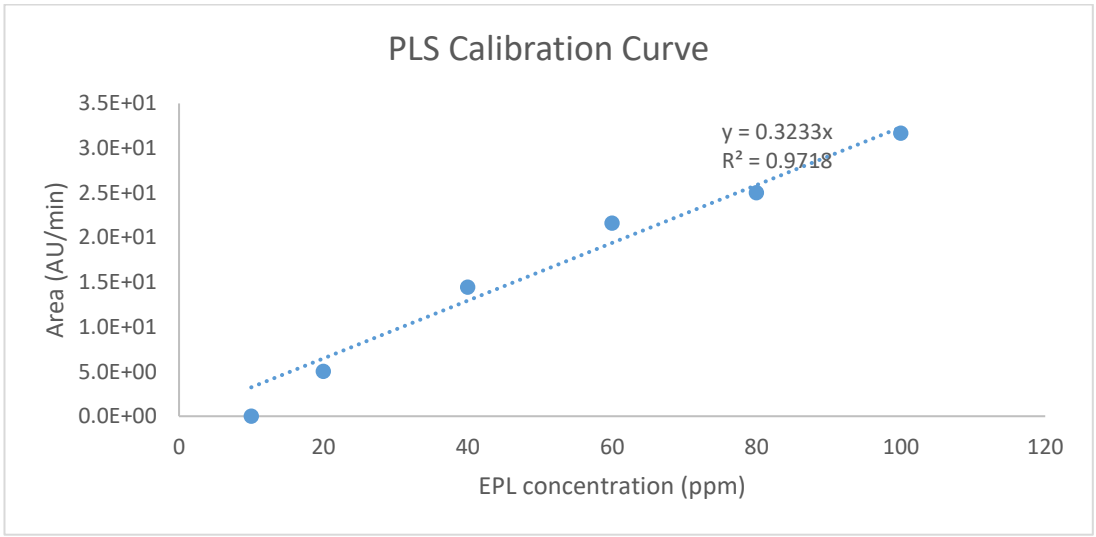

Figure 4-3: Calibration curve for Polylysine

After the calibration is completed a value is generated for the gradient which is 0.3233 $AUmin^{-1}ppm^{-1}$ and was used to calculate the polylysine present in the solution in µg/ml.

The mass, m, of EPL in the original disc is given by

$m_{EPLsample} = Pxm_t/(P+1)$

where :

x = mass fraction of EPL in the powder

m_t = total sample mass

P = powder/liquid ratio

To calculate the amount of polylysine in the solution the following formula is used

$m_{EPLsolution} = a0.3233$

where a is absorbance and is recorded in the HPLC and 0.3233 is the constant value of gradient.

To calculate the percentage of polylysine released the following formula was used

$$\% \text{ EPL} = \frac{m_{EPLsolution}\ x\ 100}{m_{EPLsample}}$$

4.7 Data Analysis

Data analysis was done using SPSS with the aid of a chartered statistician.

Data analysis was done for the bacterial viable counts, volumetric analysis and polylysine release. The *p*-value was set at 0.05. SPSS Statistics version 24 for Windows was used for statistical analysis.

Analysis of variance (ANOVA) was used to test the differences between two or more means. To perform multiple comparisons of multiple formulations the Bonferroni adjustment was used. The Levene test was performed to assess the homogeneity of variance of the samples.

5. Chapter Five: Results

5.1 Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations

Minimum Inhibitory Concentration (MIC) for polylysine and chlorhexidine were measured in 96 well plates (figure 5-1) and Minimum Bactericidal Concentration (MBC) using 12cm agar plates (figure 5-2). The objective was to determine the concentrations of polylysine and chlorhexidine required to inhibit growth or kill varying concentrations of *S. mutans* incubated in air enriched with 5% CO_2 for 24 hours (objective 1). Figures 18 and 19 show examples of MIC/MBC determination experiments.

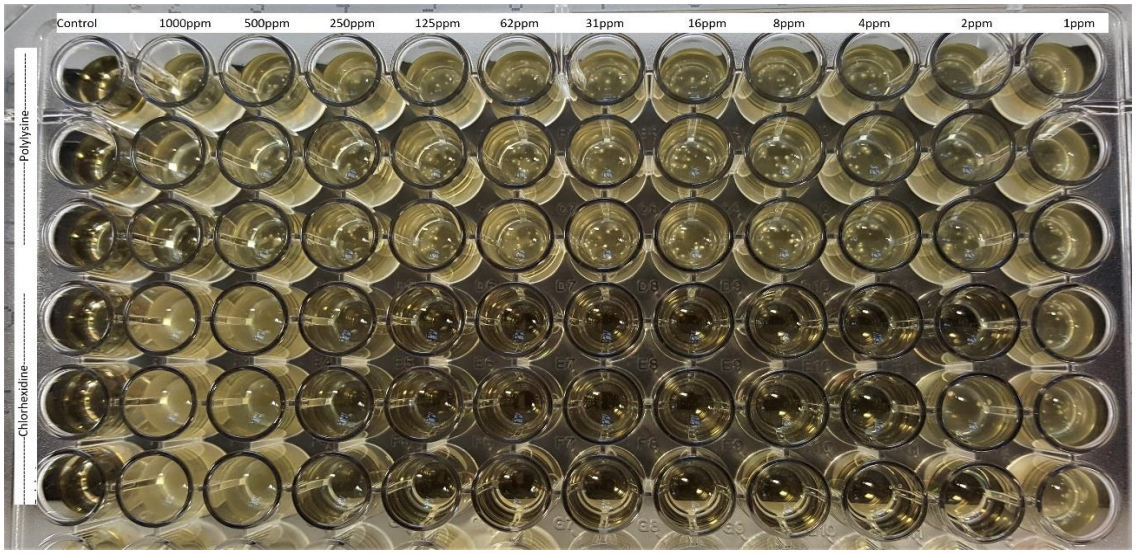

Figure 5-1: Example of a 96 well plate used to determine MIC of polylysine against S. mutans. This image is after incubation in 5% carbon dioxide for 24 hours at 37°C with an initial bacterial count of 6x10^6 CFU/ml. multiple two fold dilutions were made from 1000µg/ml to 1µg/ml. The first column is a control with no bacteria. In this example the MIC of polylysine was more than 1000µg/ml and the MIC of chlorhexidine was 4µg/ml.

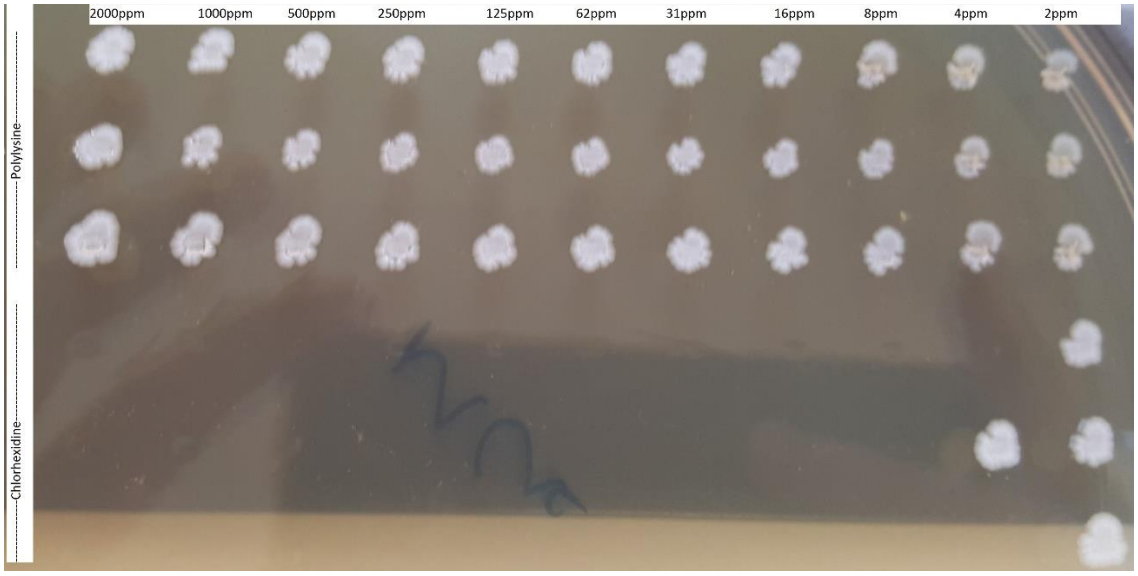

Figure 5-2: Example of an agar plate were the MBC experiment was carried out. Bacteria have grown on the areas were the metal prongs touched the agar after dipping them in the 96 well plate. In this example, the MBC of polylysine is higher than 2000µg/ml and the MBC of chlorhexidine is 8µg/mln.

The results of the MIC/MBC experiments are presented in the table below (table 5-1). As the density of *S. mutans* increased, an increase in the amount of antibacterial required to inhibit/ kill bacteria was seen. There was a significant difference between the two antibacterials with chlorhexidine being at least 100 times more effective than polylysine (objective 1).

	Polylysine		**Chlorhexidine**	
Inoculum *S. mutans*	MIC	MBC	MIC	MBC
$5x10^8$ CFU/ml	2000µg/ml	2500µg/ml	8µg/ml	16µg/ml
$6x10^6$ CFU/ml	1000µg/ml	2000µg/ml	4µg/ml	8pµg/ml
$5x10^5$ CFU/ml	500µg/ml	1000µg/ml	2µg/ml	2µg/ml

Table 5-1: Results of MIC/MBC. Two fold dilutions were made to obtain these results. 96 well plates were kept in air enriched with 5% CO_2 at 37°C for 24 hours before MIC results could be obtained. Bacteria were transferred to 12cm agar plates, which were kept in air enriched with 5% CO_2 at 37°C for 3 days before results could be obtained. Note that for Polylysine in $5x10^8$ CFU/ml a different dilution was done to obtain more accurate results. Each experiment was repeated twice and had three samples.

5.2 Novel composite formulations with added polylysine

5.2.1 Series 1 - UDMA/TEGDMA composites with MCPM/TCP and varying EPL

In the first series of composites all the components remained same apart from the Polylysine level in the glass phase which had different concentrations of 0, 2, 5, 10%. The objective was to determine the concentration of polylysine that needs to be added to UDMA/TEGDMA based composites to allow early killing of bacteria in the suspension (objective 2a).

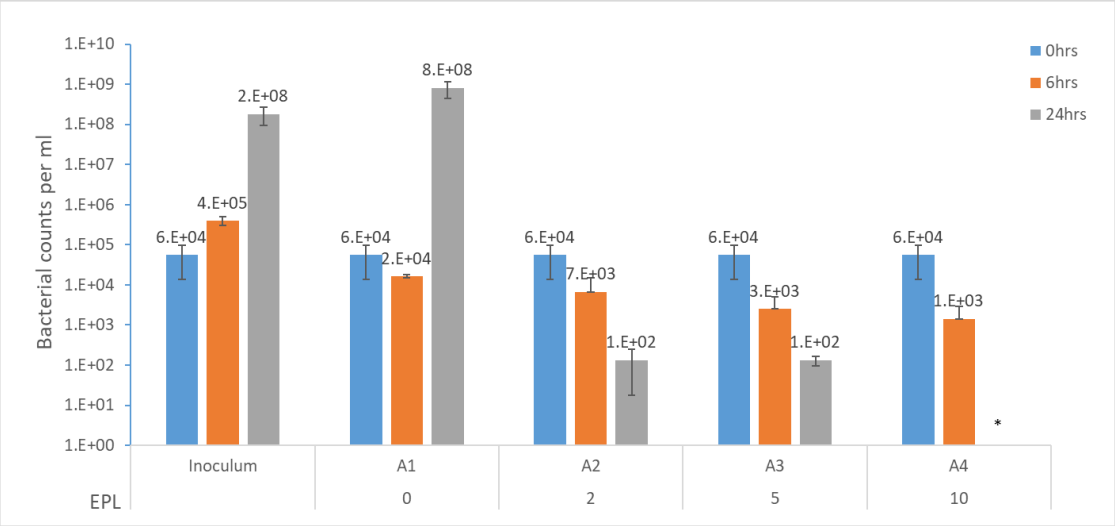

*Figure 5-3: Results for Series 1. Bacterial inoculum=6x10^4 CFU/m of S. mutans. Four different formulations with increasing concentration of polylysine were tested at 6 hours and 24 hours. Plate was kept in air at 37^{o}C while being shaken. Error bars = st dev n=3, *=0*

As seen in figure 5-3, after 6 hours, bacteria counts increased slightly in the well with no specimen from the initial density of 6x10^4 CFU/ml to 4x10^5 CFU/ml. With the composite discs however, all four formulations caused a decrease in bacterial counts of between 1 and 2 logs. The level of effect increased with increasing polylysine level. At 24 hours, the inoculum and the well containing the disc with 0% polylysine had an increase in bacterial numbers of approximately 4 log, whereas the discs with 2% and 5% EPL caused a decrease in bacterial numbers of >2 log from the initial level. The well containing a disc prepared with 10% polylysine in the glass phase had no detectable bacteria at 24 hours (limit of detection was 10 CFU/ml).

At 6 hours, there was no statistically significant difference in any of the results.

At 24 hours, the A2, A3, A4 were statistically significant different from A1 (p = 0.00 for all samples). A2 also had a statistically significant difference with A4 (p = 0.028).

5.2.2 Series 2 - UDMA and UDMA/PPGDMA composites with varying MCPM/EPL

The objective was to assess the antibacterial effect of removing or changing the diluent monomer to PPGDMA, and varying MCPM and EPL level in composites with no TCP (objective 2b). Additionally, to compare the antibacterial effect of a control composite with new monomers with those of a commercial formulation (Z250). These were tested in a suspension of *S. mutans* at an initial density of $1x10^5$ CFU/ml.

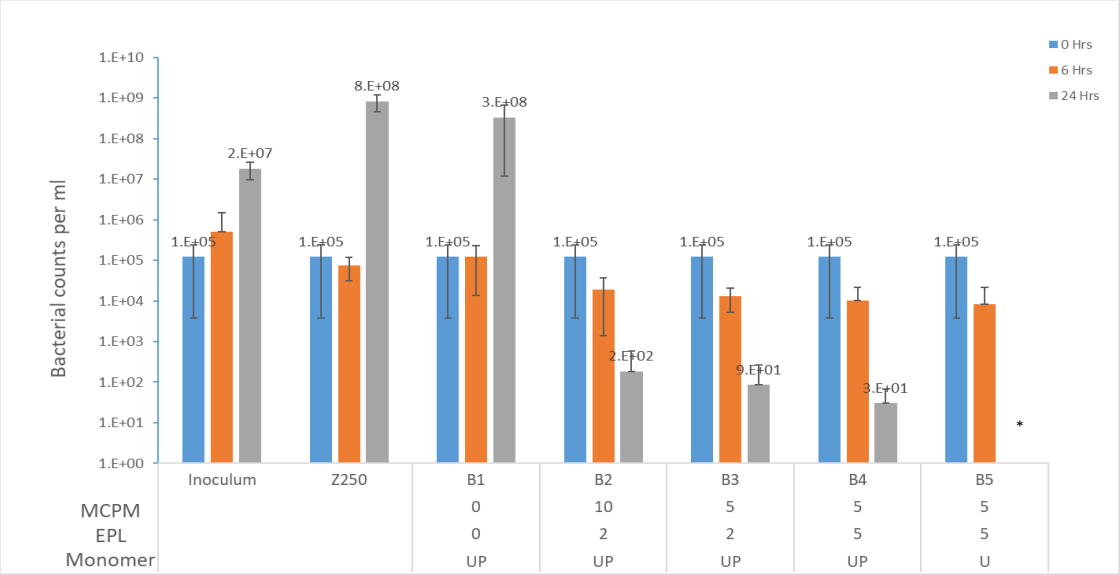

*Figure 5-4: Results for Series 2. Bacterial inoculum=1x10^5 CFU/m of S. mutans. Five different formulations and a commercial composite were tested at 6 hours and 24 hours. Plate was kept in air at 37^{o}C while being shaken. Error bars = st dev n=3, *=0*

As shown in Figure 5-4, the 0% EPL control and Z250 had no antibacterial effect. At 24 hours there was an increase in viable counts of at least 3 logs which was greater that what was seen in the inoculum at this time point. The remaining composite formulations showed a reduction in viable counts of at least 3 logs. There was some difference between the wells with composite discs with 2% and 5% EPL but it was not statistically significant.

There was no significant change in the antibacterial action upon increasing the MCPM concentration in the filler phase from 5% to 10% (B2 and B3). Furthermore, there was no significant difference when adding PPGDMA (B4 and B5) to the filler phase.

At 6 hours B1 and Z250 had statistically significant more bacteria than B2, B3, B4, B5 ($p = 0.002, 0.002, 0.002, 0.000$ and $0.011, 0.011, 0.000, 0.000$ respectively).

At 24 hours B1 and Z250 had statistically significant more bacteria than B2, B3, B4, B5 ($p = 0.000$ for all samples).

5.2.3 Series 3 - UDMA/PPGDMA composites with varying MCPM/EPL and PLR

The objective was to assess the antibacterial effect of varying PLR in UDMA / PPGDMA based formulations with poylysine as well as to compare with commercial formulations (Z250, FUJI II, FUJI IX). Additionally, to assess different environment conditions, in air shaken and in air enriched with 5% CO_2 not shaken but with 10 mg/ml sucrose added, and their effect on the antibacterial properties of composite discs with polylysine (objective 2c). The viable counts were only done at 24 hours and not at 6 hours. Figure 5-5 shows the results in air at 37ºC.

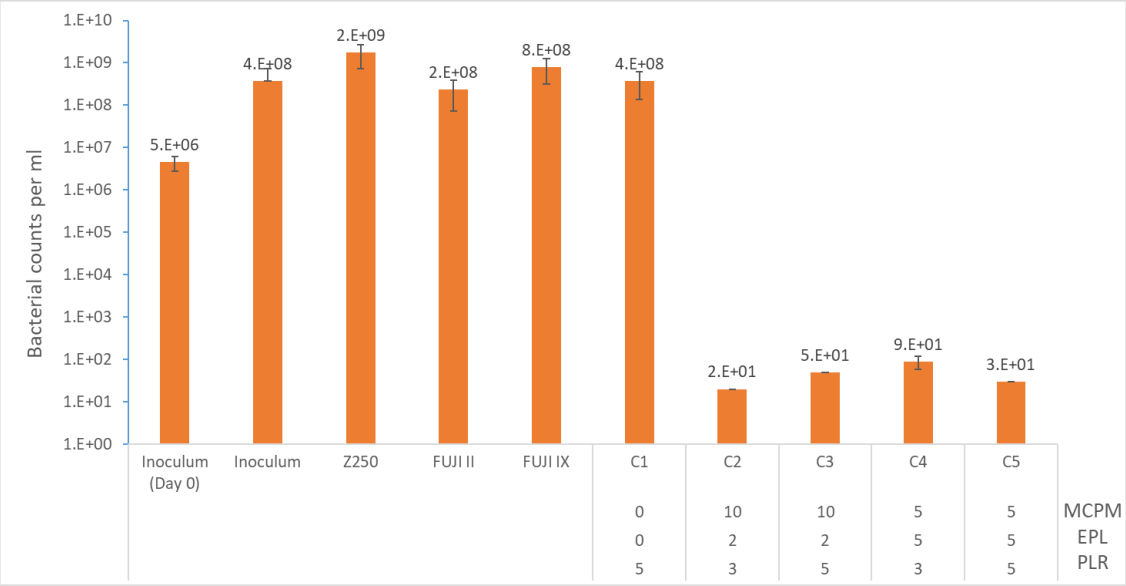

Figure 5-5: Results for Series 3. Bacterial inoculum=5x10^6 CFU/m of S. mutans. Five different formulations and three commercial materials were tested at 24 hours. Plate was kept in air at 37ºC while being shaken. Error bars = st dev n=3

In figure 5-5, the initial bacterial density was 5x10^6 CFU/ml. After 24 hours, bacterial counts increased by 2 logs in the absence of EPL. All four wells containing discs with 2% and 5% EPL showed a reduction in bacterial numbers of 7 logs after 24 hours compared to the control without EPL. The control without MPCM remained similar to the inoculum. The three commercial materials showed a 2 log increase in bacterial counts after 24 hours. The biggest increase was seen on Z250 and the smallest on FUJI II LC.

All the composite discs with polylysine had statistically significant less bacterial counts when compared with the commercial materials and the composite without polylysine (p = 0.000 when comparing C2, C3, C4, C5 with the inoculum, commercial composites and C1).

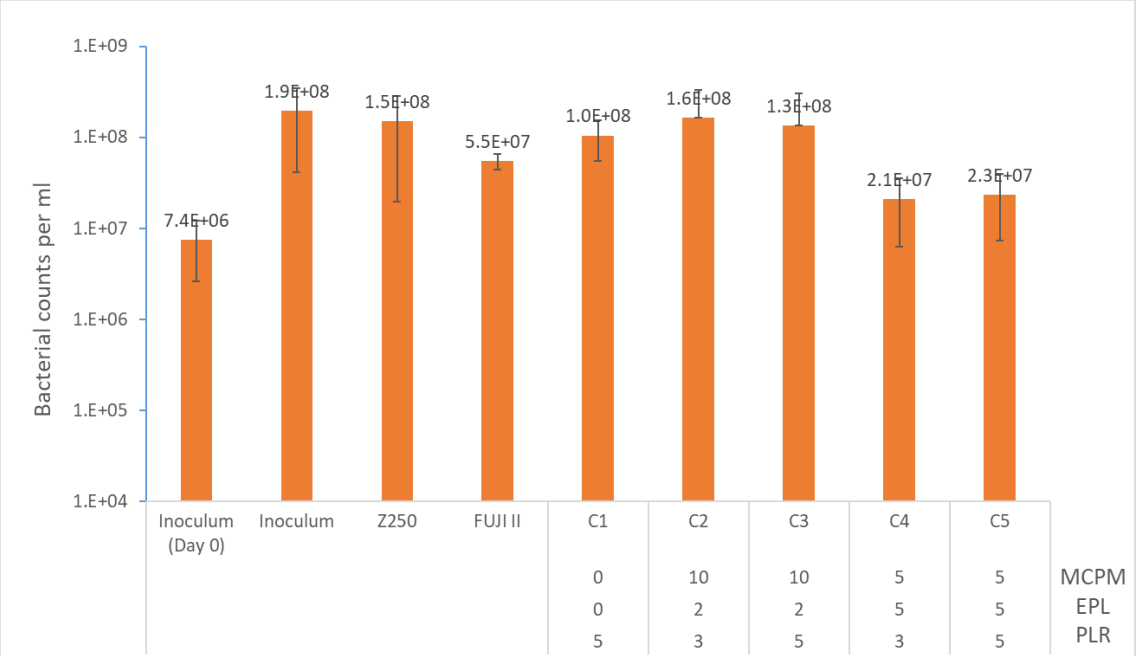

Figure 5-6: Results for Series 3. Bacterial inoculum=7x10^6 CFU/m of S. mutans. Five different formulations and two commercial materials were tested at 24 hours. Plate was kept in 5% CO_2 at 37^{o}C not shaken. Error bars = st dev n=3

In figure 5-6 the initial bacterial density was 7x10^6 CFU/ml and the experiment was done in air enriched with 5% CO_2 and added 10 mg/ml sucrose. At 24 hours, wells with the inoculum, the discs containing polylysine, the disc with no EPL and the commercial materials had increased bacterial counts of between 1 and 2 logs. The 2 wells with composite discs containing 5% polylysine (C4, C5) had a bacteriostatic effect as the increase in bacterial counts compared to the inoculum (day 0) was less than 1 $\log_{10}$.

C4 and C5 had statistically significant less bacterial counts when compared with the inoculum ($p = 0.000$ in both samples).

5.2.4 Series 4 – UDMA/PPGDMA composites varying MCPM/EPL

The objective was to determine whether a lower concentration of polylysine added to composite discs would still inhibit bacterial growth. Additionally, to assess what effect different inoculum densities have in the antibacterial properties of composite discs with polylysine. The experiment was done in air using different bacterial cell densities, $8x10^5$ CFU/ml and $8x10^6$ CFU/ml (objective 2d).

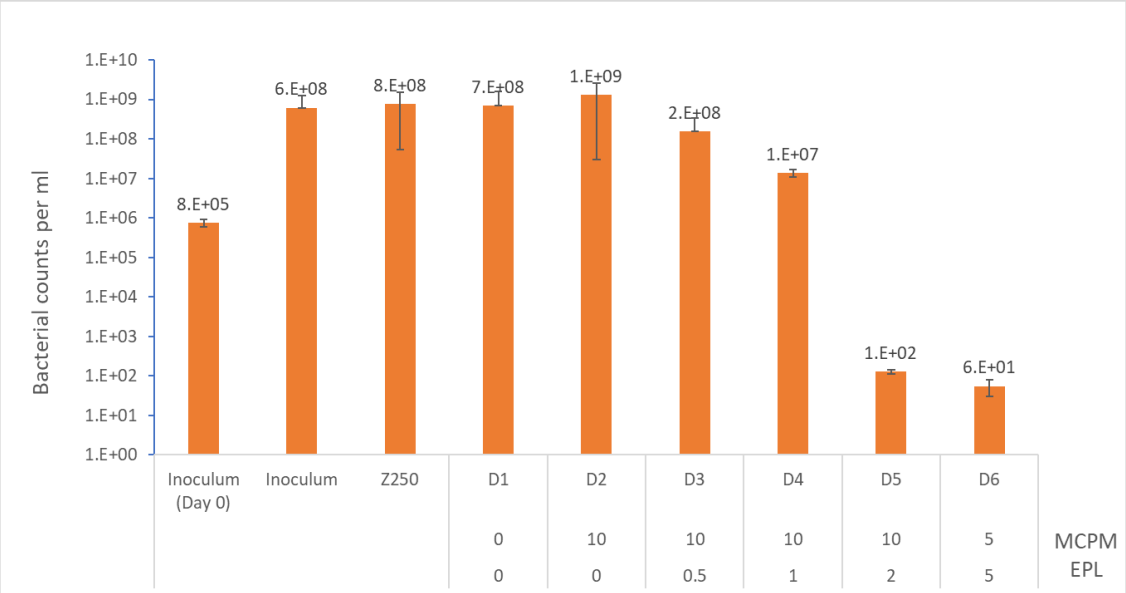

Figure 5-7: Results for Series 4. Bacterial inoculum=$8x10^5$ CFU/m of S. mutans. Six different formulations and a commercial composite were tested at 24 hours. Plate was kept in air at 37°C while being shaken. Error bars = st dev n=3

In figure 5-7, after 24 hours, both the well with the inoculum and the well containing a disc with no EPL showed an increase by 3 logs as seen in the previous experiments. The addition of MCPM 10% to the control did not have any effect on the antibacterial properties and the bacterial numbers increased by 3 logs, similar to the disc with no EPL and no MCPM. Composite discs with 0.5% EPL were ineffective as the bacterial numbers also increased by 3 logs. When 1% polylysine was added to composite discs there was a bacteriostatic effect and an increase of 1 log was seen compared to the inoculum (day 0). As in previous experiments wells with composite discs with 2% and 5% polylysine showed a significant reduction in bacterial counts of 6 to 7 logs compared with the inoculum at 24 hours.

D5 and D6 had statistically significant less bacteria when compared with the remaining composites ($p = 0.000$ for all samples). D4 had no statistically significant difference to D2 or D1 but the p-value was low ($p = 0.104$ and 0.059 respectively). D5 and D6 did not have any statistically significant difference when compared with each other.

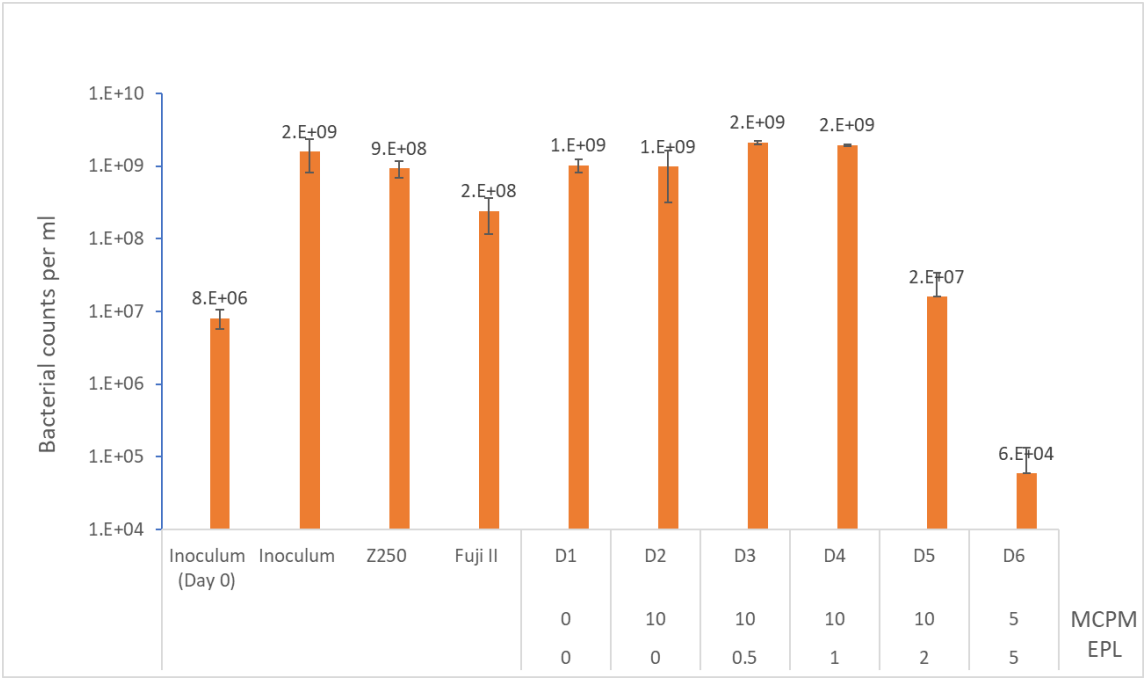

Figure 5-8: Results for Series 4. Bacterial inoculum=8x10^6 CFU/m of S. mutans. Six different formulations and two commercial materials were tested at 24 hours. Plate was kept in air at 37^{o}C while being shaken. Error bars = st dev n=3

In figure 5-8, at 24 hours, the well with the inoculum, composite discs with no EPL, 0.5% and 1% EPL and the two commercial materials showed an increase of 2 logs. The composite disc with 2% EPL showed an increase of less than 1 log and had some bacteriostatic activity. The composite disc with 5% EPL showed a decrease in bacterial counts of 5 logs after 24 hours.

D5 and D6 had statistically significant less bacteria when compared to the remaining composites (p = 0.000). Additionally D5 had statistically significant more bacteria when compared with D6 (p = 0.000)

5.3 Confocal microscopy and Scanning Electron Microscopy

For this experiment, a selection of formulations from series 4 was studied. The discs were visualised after being left in a suspension of $5x10^6$ CFU/ml *S. mutans* for three days in air or in air enriched with 5% CO_2 with 10 mg/ml sucrose.

The control and 4 different formulations were used. All had PLR 5 and all had the same monomer and glass. The EPL concentration changed from 0%, 0.5%, 1%, 2% and 5%. All formulations apart from the control and D6 had 10% MCPM. These correspond to D1, D2, D3, D4, D5 and D6 formulations.

In the images of the confocal studies, live and dead bacterial colonies can be visualised. The objective for the SEM was to assess presence of bacteria on discs and formation of a biofilm under different conditions (objective 3). The red stains on the confocal images show dead bacterial cells and the green alive. As the concentration of polylysine in the composite discs increases, an increase in the proportion of dead bacteria is apparent.

Figure 5-9 shows the images of confocal after discs were incubated for 3 days in air without sucrose.

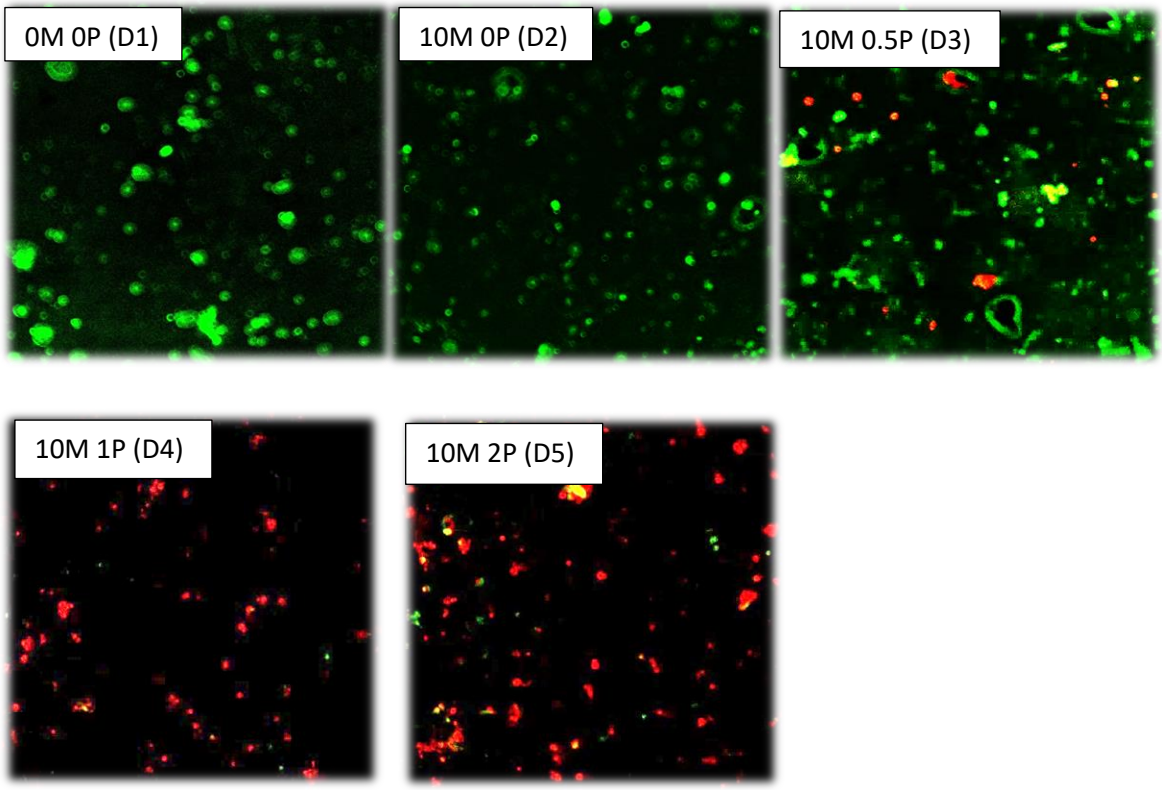

Figure 5-9:From top left to bottom right formulations D1, D2, D3, D4, D5 (M=MCPM% P=EPL%). Inoculum: S.mutans $5x10^6$ CFU/ml. Discs in suspension for three days in air before staining. Stained with Live/Dead staining. Repeats=2

In the SEM images below *S. mutans* is seen on the composite discs, however it appears as mostly individual bacteria which are not adhering to each other. The difference in concentration of polylysine in the different discs did not seem to affect the number of bacteria on the discs, however it was not clear whether these bacteria are dead or alive.

Figures 5-10 to 5-12 show discs after incubation in a suspension of *S. mutans* in air for 3 days.

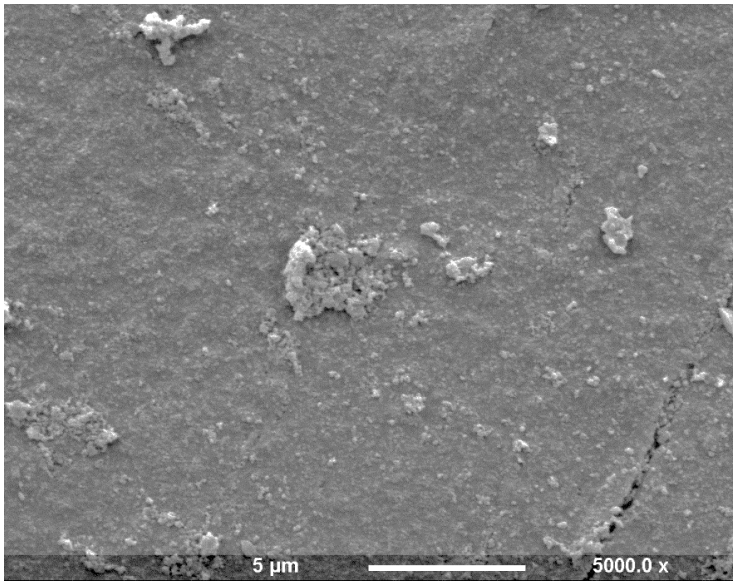

Figure 5-10: D2 (10% MCPM, 0% polylysine) formulation. Inoculum: S. mutans 5x10^6 CFU/ml. Discs in suspension for three days in air at 37^{o}C.

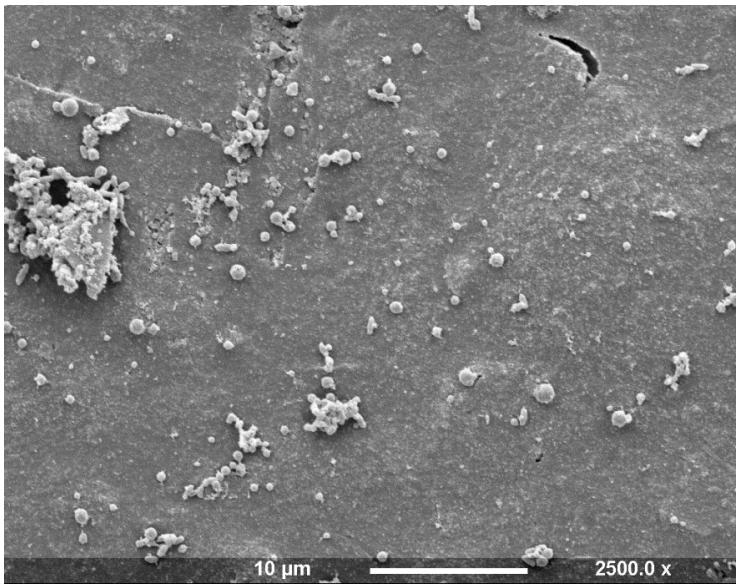

Figure 5-11: D4 formulation (10% MCPM 1% polylysine). Inoculum: S. mutans 5x10^6 CFU/ml. Discs in suspension for three days in air at 37^{o}C.

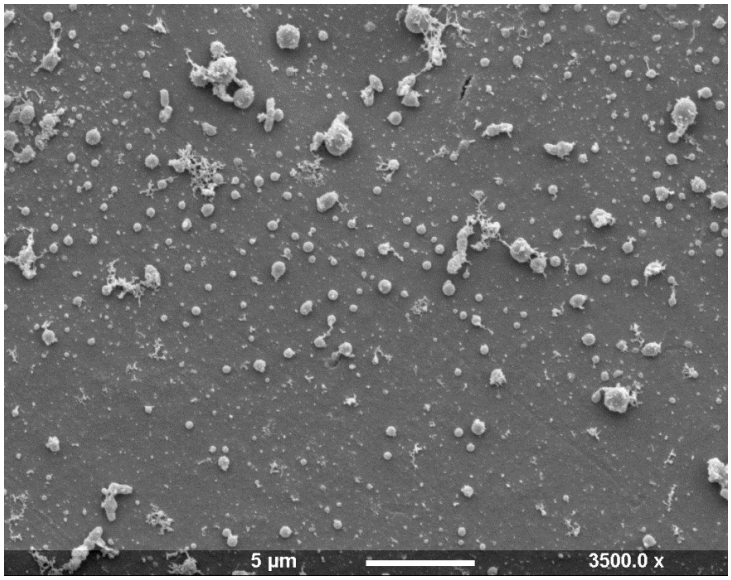

Figure 5-12: D5 (10% MCPM 2% polylysine) formulation. Inoculum: S. mutans 5x10^6 CFU/ml. Discs in suspension for three days in air at 37^{o}C.

Figure 5-13 shows images of confocal after discs were incubated for 3 days in 5% CO_2 and 10mg/ml sucrose. The proportion of dead bacteria increased as the concentration of EPL increases but some live bacteria are still present even at 5% EPL. The control discs as well as the two commercial materials allow adhesion of bacterial colonies and formation of what appears to be a biofilm on the discs. Especially in the commercial materials the biofilm covers almost all the surface of the discs.

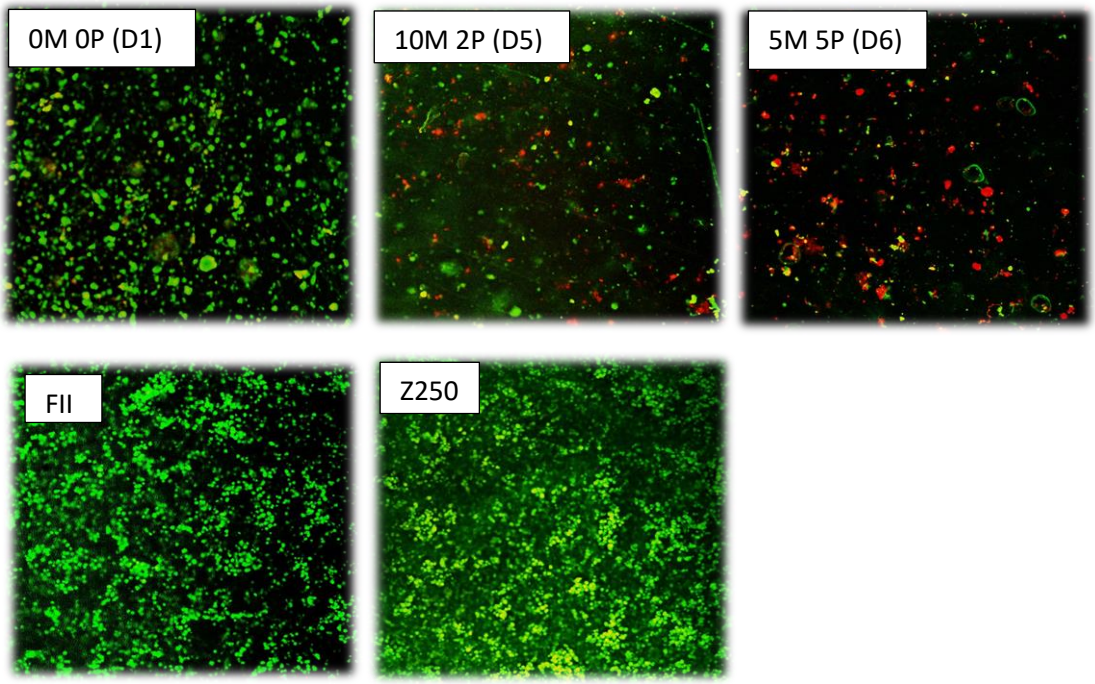

Figure 5-13: From top left to bottom right formulations D1, D5, D6 (M=MCPM% P=EPL%). Fuji II, Z250. Inoculum: S. mutans 5x10^6 CFU/ml. Discs in suspension for three days in 5% CO_2 and 10mg/ml sucrose before staining. Stained with Live/Dead staining. Repeats=2

Figures 5-14 to 5-18 show discs after suspension in *S. mutans* in air enriched with CO_2 and 10 mg/ml sucrose (objective 6). In the images below *S. mutans* was present on all discs. A very similar appearance was seen on all discs at 500x and 2000x magnification. The bacteria were adhering to each other and were forming what appeared to be a three-dimensional biofilm. An increase in the concentration of polylysine did not seem to affect bacterial adherence and proliferation on the discs. The commercial materials showed similar bacterial growth with the novel formulations.

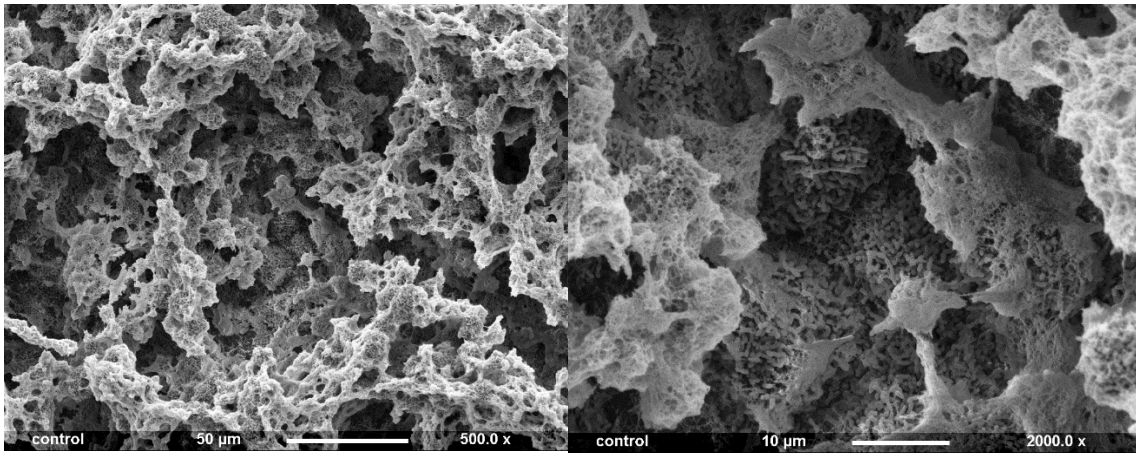

Figure 5-14: D1 (0% MCPM, 0% polylysine) formulation after three days in suspension of S. mutans 5x10^6 CFU/ml in CO_2 and 10 mg/ml sucrose at 37^{o}C. Magnification of 500x on left and 2000x on right.

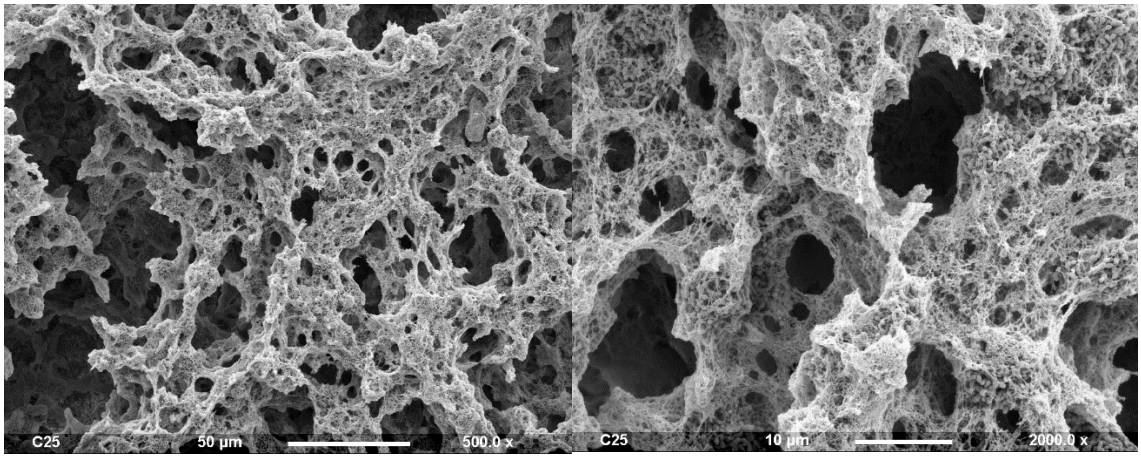

Figure 5-15: D5 (10% MCPM, 2% polylysine) formulation after three days in suspension of S. mutans 5x10^6 CFU/ml in CO_2 and 10 mg/ml sucrose at 37^{o}C. Magnification of 500x on left and 2000x on right.

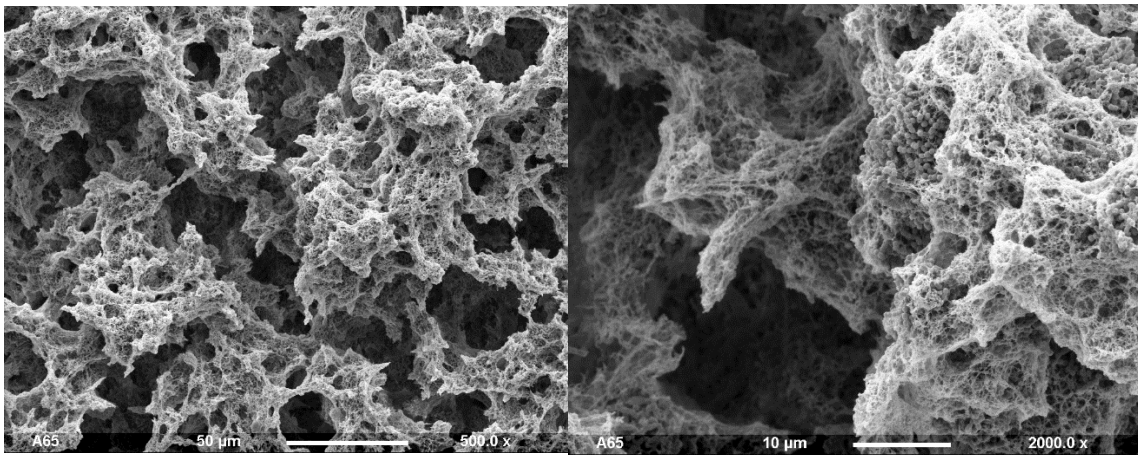

Figure 5-16 D6 (5% MCPM, 5% polylysine) formulation after three days in suspension of S. mutans 5x10^6 CFU/ml in CO_2 and 10 mg/ml sucrose at 37^{o}C. Magnification of 500x on left and 2000x on right.

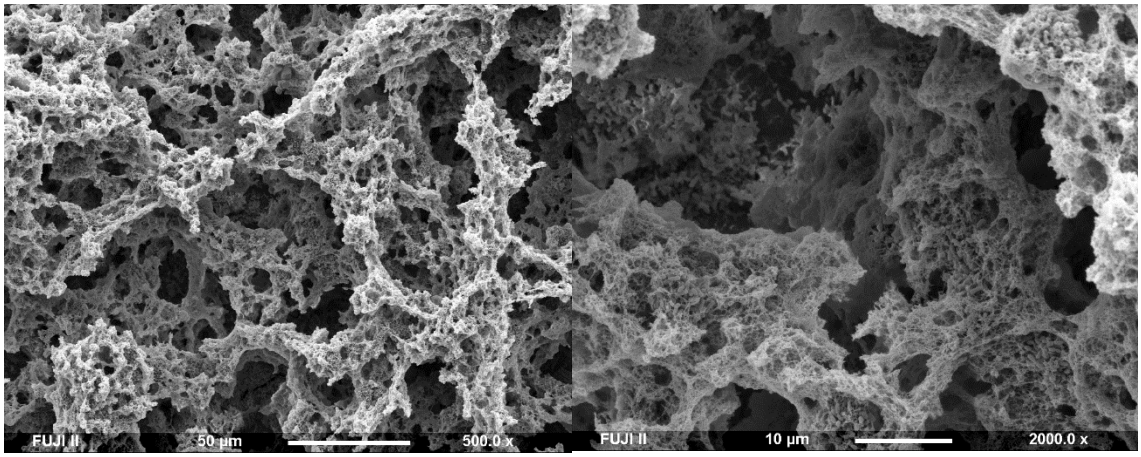

Figure 5-17: Fuji II after three days in suspension of S. mutans 5x10^6 CFU/ml in CO_2 and 10 mg/ml sucrose at 37^{o}C. Magnification of 500x on left and 2000x on right.

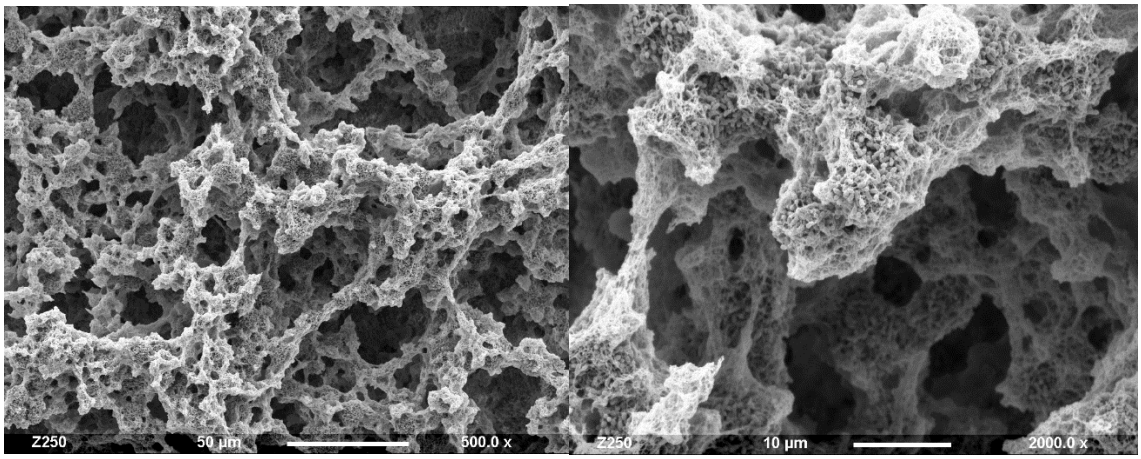

Figure 5-18: Z250 after three days in suspension of S. mutans 5x10^6 CFU/ml in CO_2 and 10mg/ml sucrose at 37^{o}C. Magnification of 500x on left and 2000x on right.

5.4 Mass and Volume

Mass and volume change over 8 weeks versus the square root of time as well as total mass and volume change are shown on graphs 5-19 to 5-23 (objective 4). The specimens were kept in deionised water at 23 degrees Celsius. Three samples were used for each formulation. In all the calculations, the 0 hour intercept has been adjusted by using the first three readings and extrapolating to zero.

Figure 5-19 showed that as the amount of polylysine increased in the formulations, the mass change over time also increased. The formulations have 0.5, 1, 2, 5% polylysine and the mass change seemed to directly correlate with the polylysine concentration.

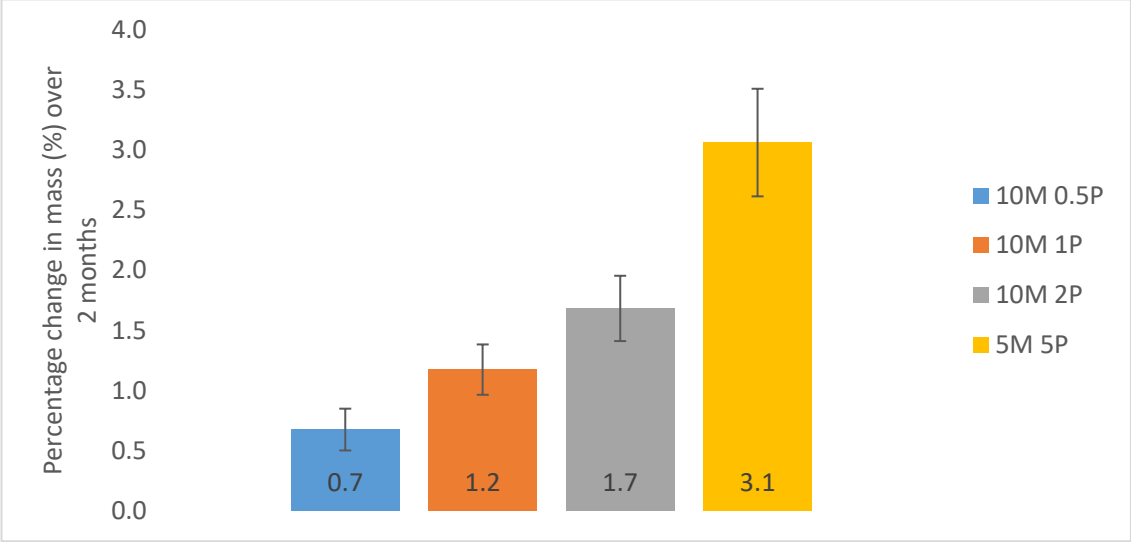

Figure 5-19: Graph of percentage change in mass of four formulations after 2 months after adjusting for the zero intercept by using the first three readings and extrapolating to 0. The formulations have all components constant apart from EPL and MCPM (M=MCPM%, E=EPL%), error bars – st. dev. n=3

10M 0.5P (D3) and 10M 1P (D4) had statistically significant less change when compared with 5M 5P (D6) ($p = 0.000$ and 0.001 respectively). 10M 2P (D5) did not have significantly more change in mass when compared to 10M 0.5P, but the p value was close to 0.05 ($p = 0.052$). 10M 2P has significantly less change in mass compared to 5M 5P ($p = 0.010$).

Figure 5-20 showed a linear increase in mass versus the square root of time for all formulations. At 2 months, final mass change was greatest for the composite with 5% polylysine at 3.1%, while the composite disc with 0.5% polylysine had the smallest increase at 0.7%. Table 5-2 shows the gradients and R^2 for figure 37. An increase in the gradient is seen as the polylysine concentration in the composite discs increases.

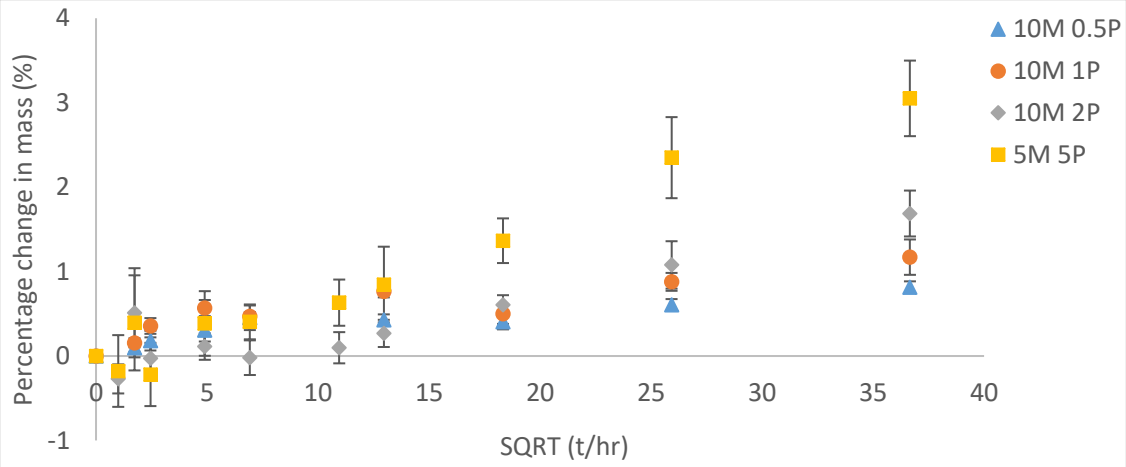

Figure 5-20: Plot of the percentage change in mass versus the SQRT of time of four after adjusting for the zero intercept by using the first three readings and extrapolating to 0. The formulations have all components constant apart from EPL and MCPM (M=MCPM%, E=EPL%), error bars - st. dev n=3

Formulations	Gradient $\%hr^{-0.5}$	R^2
10M 0.5P	y = 0.0245x	0.7023
10M 1P	y = 0.0353x	0.5971
10M 2P	y = 0.0391x	0.8054
5M 5P	y = 0.0809x	0.9526

Table 5-2: Gradient and R^2 for percentage change in mass

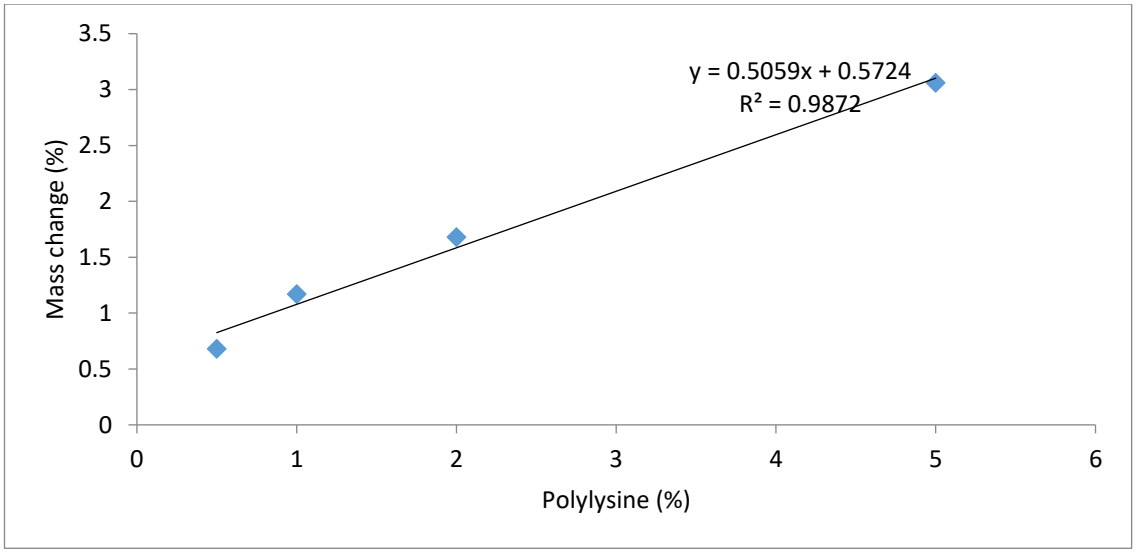

Figure 5-21: Mass change increases linearly with the concentration of polylysine.

Figure 5-21 shows the relationship between percentage mass change at 2 months and percentage polylysine in the composite discs. There is a linear correlation between the two.

As seen in figure 5-22 the volume was not affected as much by the different components with all four formulations seeing an increase of between 2.0% and 2.5% with the 5% polylysine disc having the smallest change at 2.1% and the 1% polylysine discs the largest change at 2.5% increase. None of the results have any statistically significant differences.

Figure 5-23 illustrates the initial burst change in volume before it reaches a more linear increase at approximately 10 SQRT. As seen below the volume change for all four formulations is very similar.

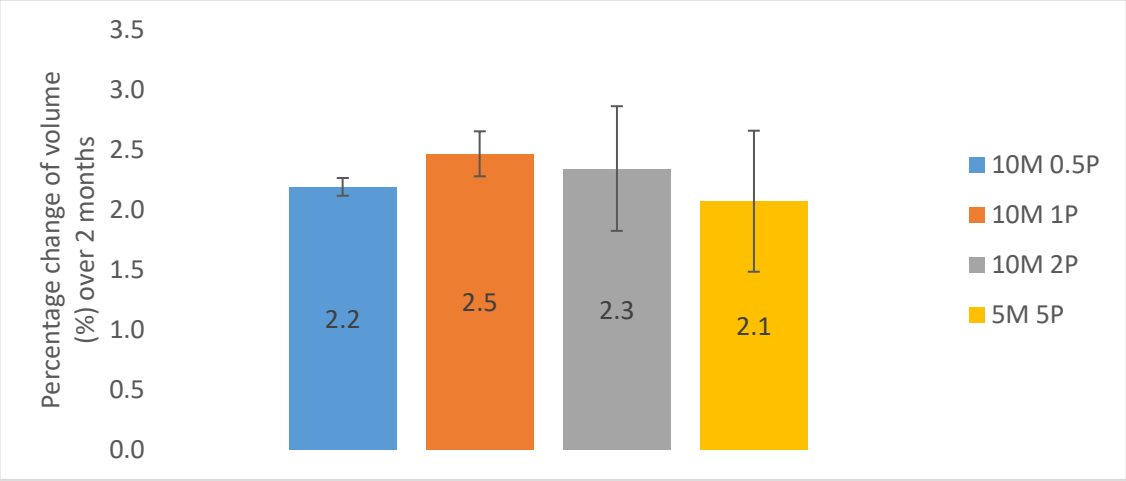

Figure 5-22: Graph of percentage change in volume of four formulations after 2 months after adjusting for the zero intercept by using the first three readings and extrapolating to 0. The formulations have all components constant apart from EPL and MCPM (M=MCPM%, E=EPL%), error bars – st. dev. n=3

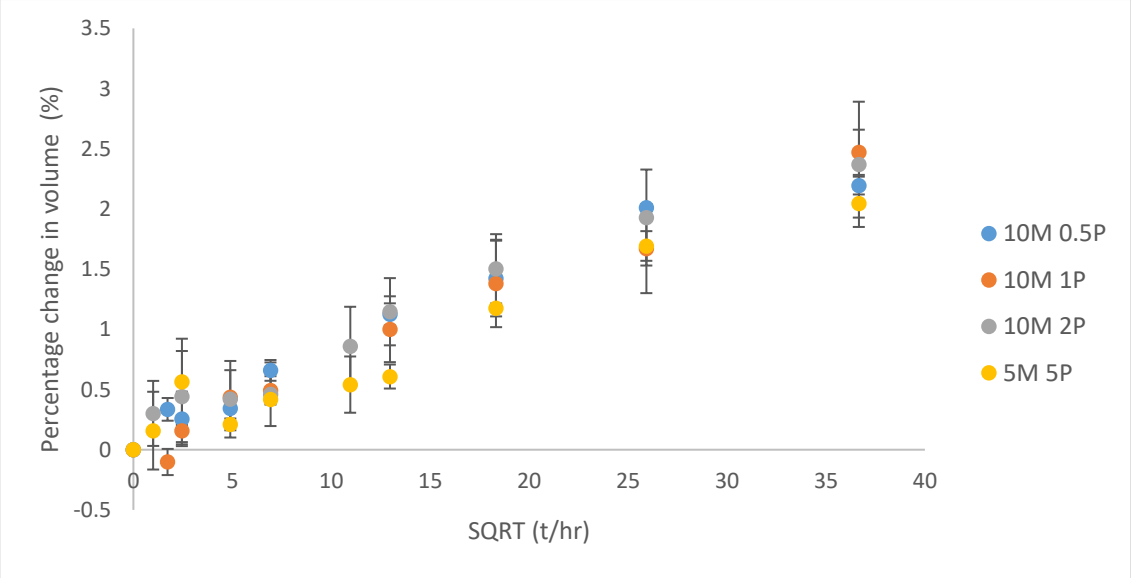

Figure 5-23: Plot of the percentage change in volume versus the SQRT of time of four formulations. after adjusting for the zero intercept by using the first three readings and extrapolating to 0. The formulations have all components constant apart from EPL and MCPM (M=MCPM%, E=EPL%), error bars - st. dev n=3

5.5 Polylysine release

A total of seven different formulations were used, same as the mass and volume experiment with the addition of D7 (0M 2P). Polylysine release was recorded at intervals for three weeks (objective 5). Three different comparisons were made. The first comparison included the four formulations with increasing polylysine content of 0.5, 1, 2, 5% (10M 0.5P, 10M 1P, 10M 2P, 5M 5P) with the remaining components being constant apart from the 5% polylysine which had 5% MCPM instead of 10%. The second comparison looked at whether adding MCPM to the formulations influences the release of polylysine. Three formulations were used, all with 2% polylysine and with 0, 5, 10% of MCPM (0M 2P, 5M 2P, 10M 2P). Finally, polylysine release in parts per million was calculated for formulations with increasing polylysine content of 0.5, 1, 2, 5% after 6, 24 hours and after 3 weeks.

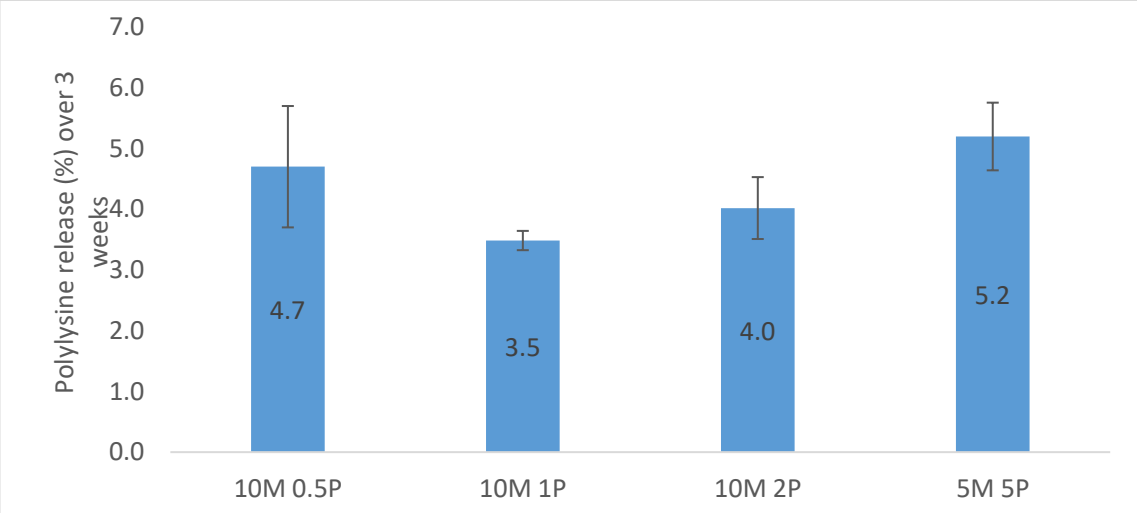

Figure 5-24: Cumulative percentage release of Polylysine over 3 weeks at 23 degrees Celsius. The formulations have all components constant apart from EPL and MCPM (M=MCPM%, E=EPL%), error bars - st. dev n=3

In figure 5-24 graph the cumulative release of polylysine can be seen for four formulations with increasing polylysine concentration. The overall percentage release is between 3.5 and 5.2% in these formulations after 3 weeks. There is a statistically significant difference between 10M 1P and 5M 5P ($p = 0.04$).

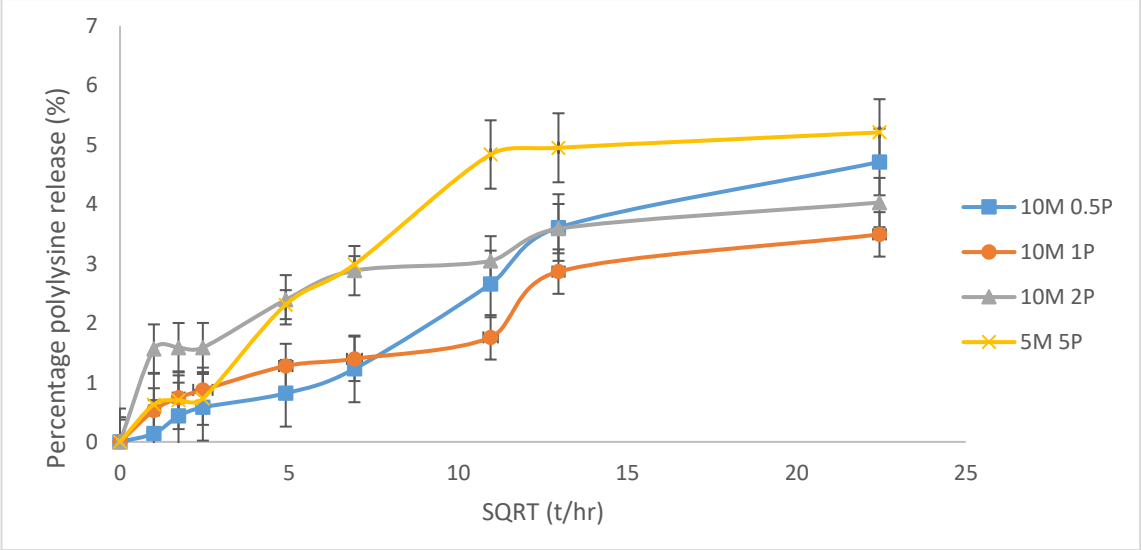

Figure 5-25: Plot of the percentage release of polylysine over the SQRT of time (3 weeks) of four formulations. The formulations have all components constant apart from EPL and MCPM (M=MCPM%, E=EPL%). error bars - st. dev n=3

In figure 5-25 the percentage release over time can be seen for the same formulations as above. There is a burst release in the first 24 hours where between 0.8% and 2.4% has already been released. Following that there is far less release over time until three weeks. The gradient and R^2 for polylysine release are presented in table 5-3.

Formulations	Gradient 7 days	R^2
10M 0.5P	y = 0.0024x	0.9513
10M 1P	y = 0.0021x	0.8593
10M 2P	y = 0.0032x	0.4585
5M 5P	y = 0.0041x	0.9819

Table 5-3: Gradient and R^2 for polylysine release

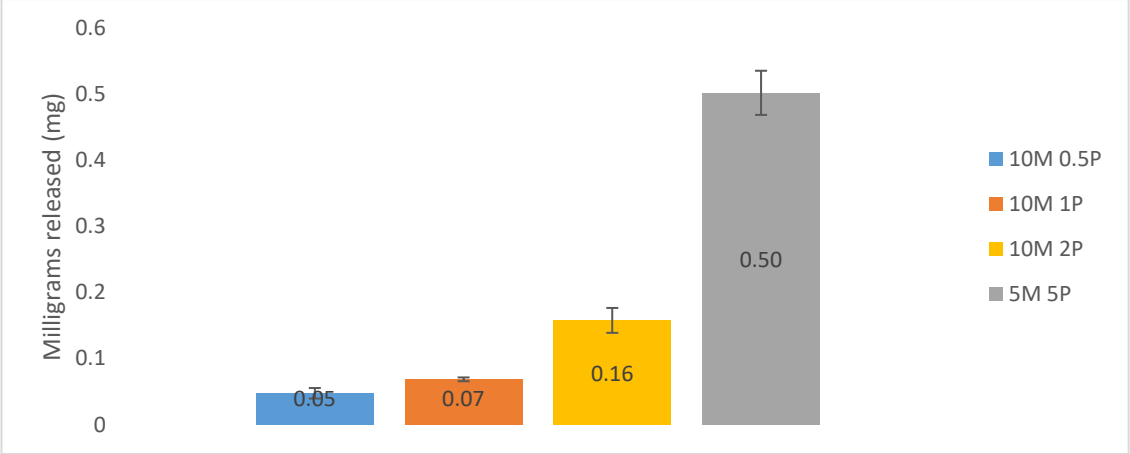

Figure 5-26: Cumulative release of Polylysine after 3 weeks in milligrams of 4 different formulations. The formulations have all components constant apart from polylysine and MCPM (M=MCPM%, E=EPL%) error bars - st. dev n=3

Figure 5-26 shows the cumulative micrograms released for the same formulations as in figure 5-24. Although the percentage release was very similar the actual micrograms released were more as the concentration increased due to the initial higher percentage of polylysine in the formulations. All compositions were statistically significant different when compared with each other apart from 10M 0.5P and 10M 1P ($p = 0.00$ for all other samples when compared with each other)

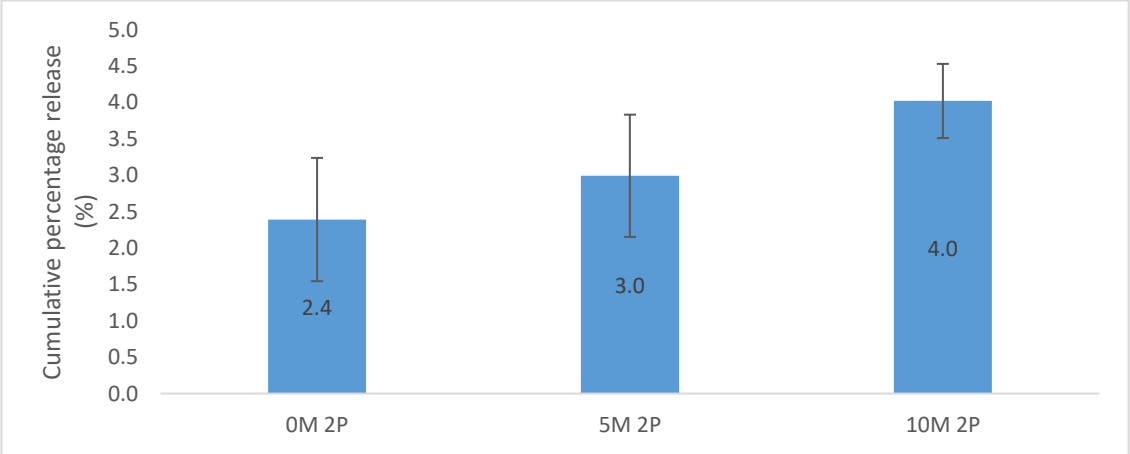

Figure 5-27: Cumulative percentage release of Polylysine over 3 weeks at 23 degrees Celsius. The three formulations have 2% polylysine and keep all components constant apart from MCPM which increases from 0% to 10%. error bars - st. dev n=3.

Figure 5-27 shows the increase in percentage release of three formulations with increasing amount of MCPM. There is a significant difference when 10% MCPM is added in the 10M 2P formulation as the cumulative increase in polylysine release over three weeks is 66% more compared to no MCPM in the 0M 2P formulations ($p = 0.016$).

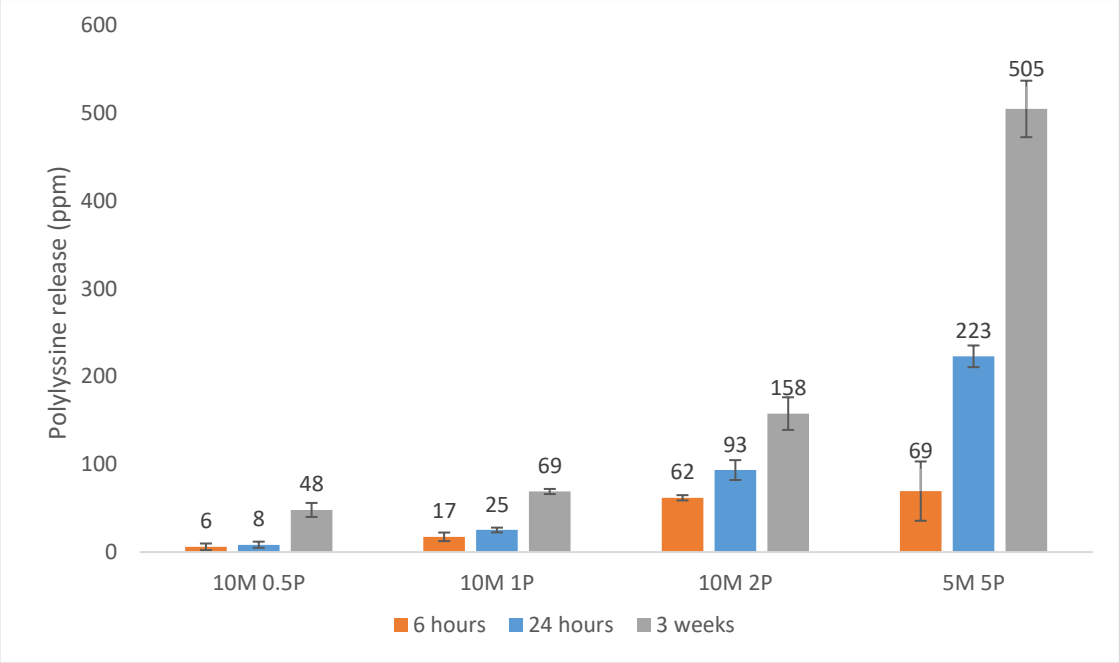

Figure 5-28: Polylysine release after 6, 24 hours and 3 weeks at 23 degrees Celsius. The formulations have all components constant apart from EPL and MCPM formulations (M=MCPM%, E=EPL%). error bars - st. dev n=3

Figure 5-28 shows the average polylysine release of the four formulations at 6, 24 hours and three weeks. The is more release of polylysine as the concentration of polylysine in the composite discs increases.

6. Chapter Six: Discussion

In this thesis, the antibacterial properties of polylysine added to novel dental composite formulations were investigated. Initially the minimum inhibitory/bactericidal concentration of polylysine on *S. mutans* was established. The bulk of this thesis concentrated on the antibacterial properties of composite discs with polylysine at different environment conditions as well as different inoculum densities. Materials were tested under different conditions attempting to mimic the aerobic conditions in the oral cavity (tested in air) as well as the anaerobic conditions in the composite-tooth interface (tested in air enriched with 5% CO_2). Additionally, the same composite formulations were investigated under microscopy (Confocal and Scanning Electron) for presence of live and dead bacteria as well as the formation of a biofilm under different environment conditions. Finally, polylysine release as well as mass and volume change of composite discs in solution over time was measured.

S. mutans is one of the most prevalent cariogenic bacteria in the oral microflora. Its association with caries is well evidenced as discussed in chapter 1.1.3 and 1.1.4. In this thesis, only one strain (UA159) of *S. mutans* was used. As discussed in chapter 1.1.4 dental caries is not caused by one single bacterial organism and is multifactorial, however *S. mutans* is one of the main causative bacteria and is especially involved in deep carious lesions. Other studies looking at antibacterial properties of dental composites also focus on *S. mutans* (Kasraei et al., 2014; Wu et al. 2015; Zhang et al., 2015). Future work should examine more strains including fresh isolates from patients' saliva rather than lab-adapted strains. Previous research has shown that it is possible to isolate *S. mutans* from saliva. Cariogenic *S. mutans* is known to be resistant to bacitracin, an antibiotic, and this can be used as a technique to isolate the bacterium from the oral microflora. It is possible to count *S. mutans* on culture plates by adding 0.2 units of bacitracin per ml of agar (McBain et al., 2005; Wu et al., 2015).

6.1 Minimum Inhibitory Concentration and Minimum Bactericidal Concentrations

The first experiment was to determine the MIC and MBC of chlorhexidine and polylysine on *S. mutans*. The reason for doing this experiment was to compare polylysine with chlorhexidine, a known potent antibacterial agent. Additionally, to be able to make a correlation between the amount required to inhibit growth as well as the amount needed to be incorporated in the composite discs to inhibit bacterial growth. In a previous study using an initial bacterial cell density of 5 x 10^5 CFU/ml, the MIC of EPL was reported as

20 µg/ml (Badaoui Najjar et al., 2009). In this work, the MIC of EPL at the same inoculum density was found to be 500 µg/ml. There were however differences in the techniques used as well as the materials that may account for the different results. The strain of *S. mutans* used was 33402 (different to the one used in this thesis), the medium used was trypticase soy broth with yeast extract and most importantly it is not stated whether the well plate was kept in air enriched with 5% CO_2 or just in air. Also, the temperature was different at 30^{o}C and the polylysine was from a different distributor. For such low results of MIC/MBC it is expected that the well plate was kept in air with no added CO_2. As seen in results of research (A. Day, unpublished) at EDI UCL, the conditions that *S. mutans* is incubated in can have an impact on its growth. Changing from air while shaken to air enriched with 5% CO_2 can increase the MIC/MBC by 10 times or more (Day, unpublished). As discussed in chapter 1.1.5, *S. mutans* uses two different pathways for adhesion with the sucrose dependent being more efficient as it allows the bacteria to adhere to each other. Additionally, being a facultative anaerobe, it can survive in oxygen but will grow more efficiently in an environment with reduced oxygen. This is the reason this thesis experiments show such a high MIC/MBC as air enriched with 5% CO_2 was used.

Chlorhexidine MIC has been previously shown to be about 1 µg/ml for 90% MIC and 0.5µg/ml for 50% MIC (Jarvinen et al., 1993). In that study 424 clinical isolates of *S. mutans* were used and 50% and 90% stands for the number of isolates inhibited at the given concentration. However in the study quoted the inoculum density is not given, although the samples were taken from saliva, which does have a density of approximately 1 x 10^{4-6} CFU/ml (Deo & Deshmukh, 2015; Pannu et al., 2013). The results in this thesis appear to be similar to the above.

It is known from the literature that chlorhexidine is a very potent antibacterial agent. Comparing it with polylysine gave an insight as to how much more potent chlorhexidine was and with some early tests it was shown that chlorhexidine could be as much as forty times more potent than polylysine. However, the limitations of chlorhexidine have been discussed in chapter 1.3.3. Even at the lowest inoculum density tested, 5x10^{5} CFU/ml, the MIC of EPL was still high at 500 µg/ml. Further research is needed to establish MIC/MBC in air at different inoculum concentrations as well as different environments. Investigating more *S. mutans* isolates, especially wild strains, would provide more information on their resistance to antibacterials such as EPL and CHX. When microorganisms grow in natural conditions, they become organised into multicellular communities that are better protected against the harmful environment. This multicellular existence allows the bacteria to differentiate and develop specific properties which will

benefit the whole community. Moving into a laboratory the bacteria can grow under more favourable conditions, which result into the suppression of some of their protective mechanisms. Organism that have been lab grown for a long time may therefore exhibit very different behaviour compared to their wild counterparts (Palková, 2004).

6.2 Novel composite formulations with added polylysine

Polylysine has been added to dental composites in previous research conducted at the UCL Eastman dental institute (Panpisut et al., 2016; Khan, 2015; Walters (a), 2016), but reports on the optimum concentration have not been made. An increased amount of EPL in the composite will have a negative effect on the mechanical properties of the material, so the minimum amount to cause inhibition of bacteria would need to be used.

The first series of formulations used constant monomer and constant glass with added MCPM, TCP and EPL. Composites with EPL inhibited the growth of *S. mutans* and even reduced bacterial numbers as the concentration increased. Overall a comparison between formulations with 0% and 2% EPL showed that the difference in bacterial counts was 6 logs over 24 hours. Generally, a 3-log reduction (99.9%) can be regarded as significant and that is also seen when statistical analysis is done where the p value is <0.05. At 6 hours, there was small difference between all the composites, which showed that the EPL level is insufficient until after the first few hours. This was further looked at when EPL release experiment was done.

Some of the components of the formulations were changed for the second series of composites. TEGDMA was replaced with PPGDMA and HEMA, DMAEMA were removed. As discussed in chapter 1.4.6, MCPM is usually combined with another calcium phosphate due to its release of phosphoric acid which can be cytotoxic in high concentrations. When TCP is added, it allows for the acid released from MCPM to be neutralised as TCP is basic. Additionally, as MCPM is hydrophilic there were concerns of high release, which would make the composite porous, and hence affect the mechanical properties. However, research done at UCL showed that MCPM reacts rapidly after initial polymerisation and does not necessarily require TCP (Dakkouri, 2015). As described above CQ usually required an amine, i.e. DMAEMA, to initiate polymerisation of the monomers. However, it has been shown that if monomers with high conversion rates are used, then DMAEMA is not required. PPGDMA and UDMA both have high conversion rates and as a result DMAEMA is not required. By removing DMAEMA, 4META also had to be reduced to allow it to dissolve. Without the amine, up

to 3% 4META can be included in the formulations as that is the highest concentration that will dissolve (Dakkouri, 2015).

The formulations from series 2 and later were adjusted due to the above findings. There was no significant difference in the antibacterial properties when adding PPGDMA to the liquid phase and when adding MCPM to the powder phase. MCPM was expected to have minimal effect as its main role is that of remineralising. Increasing the concentration of Polylysine from 2% to 5% did not produce any further antibacterial effect. At the inoculum density of 10^4 and 10^5, the 2% polylysine was enough to cause inhibition of growth of *S. mutans* in air conditions. Finally, Z250, the commercial composite by 3M showed a statistically significant increase in viable counts after 24 hours of approximately 4 $\log_{10}$. This was similar to the control composite which did not have any Polylysine.

Z250 was used as a control as it is a very common commercial composite used for posterior and anterior restorations. It does not have any antibacterial properties. As a result, when caries is left behind, these materials only rely on the bond with the tooth and for that reason the margins of the cavity have to be caries free.

In the third series of formulations, the inoculum density was further increased to approximately $5x10^6$ CFU/ml, 1 log more than in the previous set and formulations with PLR 3 were used. The conditions of the experiment were also changed. It was done in air at 37^{o}C while being shaken as before and then in an air enriched with 5% CO_2 incubator at 37^{o}C non-shaken. By changing the PLR from 5 to 3 the monomer/powder percentage was reduced from 83% to 75%. As a results EPL and MCPM (which were both present in the powder phase) would be less in the PLR 3 formulations.

Reducing the PLR to 3 and increasing the inoculum density but using a 2% EPL composite (B2) still produced an antibacterial effect in air conditions, a 7 $\log_{10}$ decrease in bacterial counts compared to control at 24 hours. In this series of composites FUJI IX and FUJI II were also tested. These glass ionomer cements contain fluoride which, as discussed in chapter 1.2.2. and 1.3.2, is known for its antibacterial properties. The antibacterial properties of fluoride are not well understood. The two glass ionomer cements saw an increase in bacterial counts after 24 hours consistent with the inoculum and with the commercial composite (Z250). There did not seem to be any antibacterial effect of the glass ionomer cements. Although there may not be any antibacterial activity, GIC's do have remineralisation properties and when placed in a carious cavity they may aid in the arrest of caries.

When the conditions changed and air enriched with 5% CO_2 was added the results were completely different. At the inoculum density of $7x10^6$ CFU/ml of *S. mutans* all the

formulations showed an increase in viable counts after 24 hours. The control, Z250 as well as the C2, C3 (2% EPL) show a 2 $\log_{10}$ increase in viable counts compared to inoculum at 0 hours. The 5% EPL formulations C4, C5 did also show an increase but less than 1 log, so there likely was some bacterial inhibition present at this level of polylysine. When comparing C4, C5 with inoculum at 24 hours there is a statistically significant difference in the viable counts. It is clear that the different environment is more favourable for the growth of *S. mutans* and as a result polylysine if far less effective and can only inhibit the growth of the bacteria at high concentrations. This can also be demonstrated in the MIC/MBC where this thesis experiments were done in air enriched with 5% CO_2 where the MIC/MBC was high, compared to previous UCL research (Dr A. Day) where the MIC/MBC was done in air and the results were much lower as the polylysine was far more effective in these favourable conditions. Additionally, when looking at Confocal images, the difference in growth of bacteria on the discs can be correlated with the viable counts experiment and the different conditions tested.

In the fourth series of formulations the aim was to use even lower concentrations of polylysine as well as re-test whether MCPM had any antibacterial effect. The experiment was also done at two different concentrations, in air, to assess how the inoculum density affects the antibacterial properties. The inoculums used were $8x10^5$ CFU/ml and $8x10^6$ CFU/ml. In this series of composites MCPM was added to the control. The 10% MCPM did not affect the antibacterial properties and the resultant density after 24 hours was similar to the control which did not contain any polylysine or MCPM. It was clear that there was a critical density of the inoculum where the polylysine stops being as effective. This was seen in this experiment, were the 1% polylysine inhibited growth at $8x10^5$ CFU/ml but was ineffective at $8x10^6$ CFU/ml inoculum density. Similarly, the 2% polylysine reduced bacterial counts at $5x10^6$ CFU/ml (tested in third series of formulations) but when the density increased it only inhibited bacterial growth at $8x10^6$ CFU/ml. In these conditions, if the growth of *S. mutans* has already been inhibited, increasing the concentration further would reduce the viable bacterial counts. This is because the inhibitory concentration is always equal or less than the bactericidal concentration. A further increase in polylysine concentration would not have any additional effect. It did seem then that through these experiments the 2% polylysine always had some antibacterial effect even at higher densities of inoculum and that is the critical concentration of polylysine required when tested under air conditions.

The aim of this experiment was to see if the addition of polylysine to the novel composite formulation would affect bacterial growth in a simulated environment where the discs were constantly shaken at 200rpm at 37^{o}C in air condition or non-shaken in air enriched

with 5% CO_2. This may simulate the restoration in the oral cavity where the tooth constantly being flushed with saliva/food/toothpaste and the interface below the restoration. Shaking also prevents the formation of a biofilm. Biofilm is very important for the growth of *Streptococcus mutans.* When the experiment was done in air enriched with 5% CO_2 conditions and sucrose, *S. mutans* grew very quickly as the conditions would be very optimum for its growth. This was shown with the third series of composites were the same formulations with the same inoculum showed very different properties in air and in air enriched with 5% CO_2. *S. mutans* is far better at proliferating in the presence of CO_2 and for that reason composite discs with polylysine were far less effective at inhibiting its growth. Additionally, due to the formation of a biofilm it was much more difficult for polylysine to penetrate through the biofilm and inhibit growth. However, as the composite/dentine interface underneath the restorations has reduced oxygen, there is an argument for also testing the composite discs in air enriched with 5% CO_2. All the commercial materials, Z250, FUJI II LC, FUJI IX, saw an increase in bacterial counts after 24 hours. This was all done under the same conditions.

Handling of materials

Manual handling of materials for preparation of composite discs showed that formulations without diluent monomer (PPGDMA, TEGDMA) and PLR of 5 proved to be very difficult to handle. In a clinical scenario, these formulations would not be used. The addition of PPGDMA improves the handling and flowability significantly and as a result this was similar to commercial materials such as Z250 when the PLR is 5. Formulations with a PLR of 3 were quite flowable and as a result they were difficult to handle with instruments due to the stickiness. However, when used with a delivery system to place directly on the tooth their handling was much improved. Their flowability was similar to FUJI II LC. Using a PLR 3 would be more suitable for a cavity which has not been excavated and caries has been left behind. This is because the flour of the cavity will be uneven and the composite will flow better on it. Only the presence of a diluent monomer and the PLR affected the handling properties. Other components such as polylysine and MCPM did not have affect the handling of the materials.

Limitations

In this thesis, bacterial counts were done from the suspension were the composite discs were placed. When the discs were kept in air for 24 hours there was no biofilm formed and only planktonic bacteria were present on the disc (as seen in the Confocal/SEM). As a result, bacterial counts from the suspension also represented the number of bacteria on the composite discs. However, when the discs were placed in CO_2 with added sucrose

a biofilm was formed. When a biofilm had formed there would be a number of bacteria attached to the disc, which were not counted. Attempts to remove a three-day biofilm and count the bacteria proved to be unreliable in this research. The discs were gently washed and placed in a sterilin tube with 1ml of BHI, then 2 glass beads were added and the tube was vortexed for 10 seconds. Bacterial counts done after the above procedure were not reliable and the results were inconsistent. As a result this technique was not used. In other studies a similar technique, whereby a 2-day biofilm of human saliva on a composite disc was harvested by sonication and vertexing was employed, seemed to be reproducible (Wu et al., 2015; Zhang et al., 2015). In this study, removing the biofilm after 2 days was not attempted. Additionally, staining the discs with Crystal violet (CV) stain was attempted, however this didn't provide reproducible results either. The reason was that addition of alcohol to attempt to remove the CV from within the cells was unsuccessful, likely due to the dense biofilm that had formed on the discs.

As discussed before this experiment was only done under certain conditions and only with one bacterium, *S. mutans*. Although the results are conclusive, a more clinical scenario would be to test composite discs placed into tooth cavities and then immersion in a suspension of saliva. This experiment would represent the oral cavity much closer.

6.3 Confocal microscopy

The aim of using Confocal was to visualise the bacterial growth on the composite discs. It is understood that bacteria do not only exist in a planktonic state, where they float and swim in a liquid medium but also can attach to the composite discs and possibly form a biofilm. As discussed in chapter 1.1.5 *S. mutans* requires sucrose to synthesize polysaccharides that will form a biofilm matrix and allow it to attach to surfaces as well to each other. For that reason, 1% sucrose was added to the suspension of *S. mutans* to not only allow it to replicate faster but also to attach to the discs and form a biofilm. Additionally, materials were tested under different conditions attempting to mimic the aerobic conditions in the oral cavity as well as the anaerobic conditions in the composite tooth interface. Growth under air enriched with 5% CO_2 conditions was expected to be greater due to *S. mutans* being a facultative anaerobe. A biofilm was only formed under these conditions and only on the control and the commercial materials. What could be seen on both experiments was a greater proportion of dead bacteria as the concentration of EPL increased. It was also evident that as the environment changed the results for the same material under different conditions changed significantly. The control only seemed to have planktonic bacteria on the disc under air but when 5% CO_2 was

introduced there was a biofilm with almost all the disc covered with bacterial colonies. The composite discs with 2% EPL reduced bacterial counts under oxygen but when under carbon dioxide conditions there was almost a 50% split between live and dead bacteria. The result was the same for the composite discs with 5% EPL formulations. What was the most interesting observation of this experiment was the vast increase of bacteria on the discs on the commercial materials compared to the composite discs 5% EPL composite formulation under anaerobic conditions.

6.4 Scanning Electron Microscopy

Scanning Electron Microscopy was also used to visualise biofilm formation on different composite discs incubated in different conditions. The aim was to investigate under what conditions a biofilm could be formed on the composite discs and whether there was any effect of the added polylysine to the formation of a biofilm. SEM was used on the discs following three days incubation in air or in air enriched with 5% CO_2 in an inoculum density of $5x10^6$ CFU/ml. Only planktonic bacteria were visible when incubated in air, and in occasions there were no bacteria visible at all. Under air enriched with 5% CO_2, growth of what appeared to be a biofilm on the discs had completely covered the composite discs. Bacteria adhered to each other and formed a three-dimensional structure surrounded by polysaccharides. It appeared that by using Confocal which only 'sees' at one plane it was not possible to visualise the full depth of the biofilm when that had grown. At the same time, having a biofilm formed did not mean that some of the bacteria on it were not dead. On the other hand, under air conditions only scattered planktonic bacteria were seen on the discs.

When trying to relate the SEM with the Confocal images it seemed that under air there was always going to be a similar number of planktonic bacteria on the discs, but whether these were alive or dead depended on the amount of polylysine used. Under air enriched with 5% CO_2 and 1% sucrose all the discs had what appeared to be a biofilm formed but when comparing with the confocal the bacteria were not as dense on the 2% and 5% polylysine formulations. This could be explained by the fact that only one plane of bacteria was seen with Confocal and this plane is usually the most superficial. At this plane polylysine had probably killed some of the bacteria and these were visible in the Confocal.

6.5 Mass and Volume change

Dental restorations are exposed to fluids from the oral cavity continuously. Any dental composite will have micro voids after the setting reaction has taken place. When immersed in solution (i.e. deionised water) over time the water will be absorbed by the voids in the composite and the mass will increase. The volume however will remain the same. Water will also be absorbed by the disc and cause swelling which will as a result increase the volume and the mass. The expanded through water sorption polymer network results in reduction in mechanical and physical properties (Sideridou et al., 2007). Water sorption is however required for components to be released (Zhang et al., 2016). The composite disc will be releasing components, mainly calcium phosphate and polylysine leading to a decrease in mass with the volume again remaining unchanged. Due to the differences in density of the components the mass and volume change will also be proportionately different as water has a density of $1g/cm^3$ whereas the composite disc will have density of approximately $2g/cm^3$. For that reason, if water replaced components release from the composite discs, the volume change of the samples is expected to be approximately double of that of the mass change.

The dissolution of MCPM produces phosphoric acid and dicalcium phosphate. Phosphoric acid acts as etchant to enhance bonding and dicalcium phosphate will bind with water after it precipitates as lower density brushite.

As MCPM and EPL are both hydrophilic, an increase in mass in the early stages was seen as expected, due to the increase in water sorption.

This thesis studies indicate that an increase in the content of polylysine will have an effect on the mass by increasing it. The increase in mass is relatively proportional with the increase in polylysine content, with the larger increase seen when 5% polylysine is added to the formulations. This suggests that EPL can improve the precipitation as seen in previous research (Panpisut et al., 2016).

The volume of the composites increases through the water expanding the polymerized resin matrix (Mehdawi et al., 2013). As seen already, polylysine will affect mass change, however its effect on volume is much less evident.

These results coincide with previous studies and also are similar with other commercial composites indicating that temperature may not affect the mass and volume change significantly (Panpisut et al., 2016; Wei et al., 2013). In this experiment, the discs were kept in distilled water at 23ºC whereas in other studies they were kept at 37ºC.

6.6 Polylysine Release

Polylysine is an antibacterial agent that has not been used in dental composites before. It is an excellent alternative to chlorhexidine, which has been the most commonly used antibacterial agent in composites. It overcomes the issues of chlorhexidine hypersensitivity reactions and antibiotic resistance (Pemberton & Gibson, 2012). Polylysine is highly soluble in water and for that reason it can in itself encourage water sorption. In this thesis experiments, an increase in polylysine release was seen as the concentration of polylysine in the formulations increased. Although the percentage release remained similar over the different formulations, (between 3.5% and 5.2%) the milligrams released where proportionally higher as the polylysine percentage increased. This was expected as the more polylysine the composites had the more the release. Previous experiments have shown a much higher release over time from a 2.5% EPL formulation, approximately 5.5% after 24 hours and 9% at 3 weeks, these however were carried out at a different temperature, hence the diffusion coefficient was different. Additionally some of the components of the materials were different, the powder phase also had TCP and the liquid phase had TEGDMA instead of PPGDMA (Panpisut et al., 2016).

However, the conclusion that increasing the polylysine content will increase its release can still be made. This will have beneficial effect on the antibacterial activity of the composite formulations. In our experiments, up to 2.4% of polylysine was released in the first 24 hours when after 3 weeks this had doubled to 5%. This early high release of polylysine may be able to inhibit further growth of bacteria in the composite/tooth surface.

By comparing the three formulations of 2% polylysine, each with 0%,2% and 10% of MCPM, it was evident that MCPM aids the release of polylysine. Up to 66% increase of polylysine release was seen when 10% MCPM was added to the formulation compared to 0%.

The release of polylysine in the current study can be explained using the modified diffusion equation below

$$\Delta EPL = \Delta EPL_0 + \Delta EPL_\infty \sqrt{\frac{2Dt}{\pi d^2}}$$

Where ΔEPL; the change in cumulative EPL in the solution, ΔEPL_0; early burst release, ΔEPL_∞; maximum change in the solution, *D*; EPL diffusion coefficient, *t*; time, *d*; sample thickness.

After 24 hours, the D4 (10M 1P) discs had an average (n=3) of 25.2 µg/ml of polylysine released and the D5 (10M 2P), D6 (5M 5P) had much higher release (93 µg/ml and 222 µg/ml respectively) whereas the D3 (10M 0.5P) had only 8.5µg/ml released. This could show a correlation between MIC/MBC in air or in 5% CO_2 and the percentage of polylysine in discs required to inhibit bacterial growth. This thesis MIC/MBC studies were done in 5% CO_2 with the MIC at $6x10^6$ CFU/ml being 1000 µg/ml. This explains why in viable counts experiment in 5% CO_2 there is bacterial growth even with the 5% formulations as the µg/ml released are far less than 1000 µg/ml. However in the study by Najjar et al the MIC at $5x10^5$ CFU/ml in what we assume to be air was only 20 µg/ml (Badaoui Najjar et al., 2009). This could explain why the D4, D5, D6 (10M 1P,10M 2P, 5M 5P) formulations inhibit growth in air conditions as they release more than 20 µg/ml of polylysine, where the D3 formulations (10M 0.5P) which released less than 20 µg/ml did not inhibit growth. Even at 6 hours composite discs with 2% and 5% EPL had more than 20 µg/ml of polylysine released, which explained the inhibition seen in the series one and two formulations which were tested at 6 hours and 24 hours. Further work on this field is required.

The conclusion that increasing the polylysine content will increase its release can be made. This will have beneficial effect on the antibacterial activity of the composite formulations. Additionally, the early release of polylysine may be able to inhibit further growth of bacteria in the composite/tooth surface.

6.7 Clinical Relevance

In the context of caries management as discussed in chapter 1.1.6, materials with antibacterial properties may be able to inhibit the growth of dental caries related bacteria that have been left behind in the cavity or not removed at all in techniques such as partial caries removal or atraumatic restorative technique. These composites may be able to prevent bacterial growth at the tooth-composite interface, at the margins of the restoration as well as below the restoration. In combination with its remineralising properties, this will allow placement of the novel composites in tooth cavities that have not been excavated. Additionally, the PLR of these composites is low, increasing its flowability and allowing placement in unprepared cavities. Furthermore, as these composites do not require the use of ‘etch and bond’, they can be placed in one step, which further simplifies the procedure of restoring a tooth. Specifically, in young patients were cooperation is limited as well as in primary teeth the application of these composites may be ideal. They may be able to replace materials such as resin modified glass

ionomer cements, composites and preformed metal crowns in carefully selected cases. The novel composites discussed in this thesis have promising characteristics for minimally invasive tooth restorations.

7. Chapter Seven: Future work and Conclusion

Future work

In this thesis, the antibacterial properties of composite formulations containing polyline were investigated in vitro.

Considering the current experiments, changes could be made to the existing methods. MIC/MBC should be investigated under air after 24 hours instead of under air enriched with 5% $C0_2$ after 24 hours. Different bacteria that cause dental caries may be investigated such as *Streptococcus sobrinus* as well as *Lactobacillus* spp. Additionally wild isolates of *S. mutans* should be looked at.

The antibacterial properties of composite formulations with polylysine should be investigated for other dental caries related bacteria as well as different *S. mutans* isolates. Additionally, human saliva can be used as an inoculum to assess how effective polylysine is on the oral microflora. A technique for acquiring human saliva for these tests has been described before (Wu et al., 2015; Zhang et al., 2015).

For microscopy with SEM and Confocal, other caries related bacteria should be investigated. Lower densities of *S. mutans* should be looked at to determine if there is a concentration of polylysine that prevents biofilm formation under air enriched with 5% CO_2 conditions.

Additionally, further work is required to make a correlation between MIC/MBC, viable bacterial counts and polylysine release from discs at different environments and inoculum concentrations. All the above will contribute in the decision on the final formulation before production.

Considering further work on the antibacterial properties of the novel composite formulations, they should be placed in extracted teeth with carious cavities and these should be immersed in suspensions of human saliva. Following that bacterial counts can be done similarly to this thesis methods as well as investigation of the tooth/composite interface with microscopy. This would give a closer impression to the oral cavity of how the composite formulations perform. Finally, once a final formulation has been decided in vivo studies can commence.

Conclusion

Traditionally dental caries has been managed with complete caries removal and restoration. This is a well evidenced and effective way of dealing with dental caries (Ricketts et al., 2013). It is however on many occasions impractical to do this in young children due to limited cooperation. For that reason, in recent years the focus of dentistry has shifted towards preventions and minimally invasive dentistry. The concept of sealing caries is now well evidenced (Tellez et al., 2013).

The goal for our team of researchers is to create a dental composite that will have all the mechanical properties of commercial composites but also be able to bond directly to enamel/dentine as well as have antibacterial and remineralising properties. This thesis focuses on the antibacterial properties.

This thesis concentrated on *Streptococcus mutans,* the main bacterium involved in dental caries, and polylysine, an antibacterial agent that was used in the composite formulations. Through a series of experiments, it was determined what concentration of polylysine inhibits bacterial growth in solution (objective1) and when placed in different dental composite formulations (objective 2). The experiments were done in different density of the inoculum and in different conditions. Addition of MCPM and PPGDMA did not affect the antibacterial properties of the composite formulations. It was concluded that the addition of 2% polylysine to the novel composite formulations will inhibit bacterial growth in air after 24 hours at all inoculum concentrations used. When tested under air enriched with 5% CO_2 and 10mg/ml sucrose, only composite discs with 5% polylysine were able to have some limited antibacterial activity. This is in agreement from the experiment in solution where the Minimum Inhibitory Concentration of polylysine on *S. mutans* was 500 μg/ml at $5x10^5$ CFU/ml in 5% CO_2 but when tested in air this can be up to 25 times less at 20 μg/ml at the same concentration (Badaoui Najjar et al., 2009). The commercial materials, Z250, FUJI II, FUJI IX, did not exhibit any antibacterial properties.

Composite discs were visualised under Confocal after Live/Dead staining and SEM (objective 3). With the Confocal, more dead bacteria were seen on the discs as the concentration of polylysine in composite discs increased from 0.5% to 5% EPL. With the SEM, a formation of what appeared to be a biofilm on the composite discs was seen under air enriched with 5% CO_2 conditions with added sucrose in all formulations tested. In these conditions, there was no difference in the growth of bacteria on all formulations and commercial materials. Under air only planktonic bacteria were visible on the discs.

Mass increase over two months at 23^{o}C seemed to be affected by amount of polylysine in the formulation whereas volume was not affected. The higher the concentration of polylysine the higher the mass change (objective 4).

There was significantly higher polylysine release in formulations with higher polylysine concentration, from 0.05mg to 0.50mg as the polylysine concentration increased from 0.5% to 5% after three weeks (objective 5). The addition of MCPM allowed for more release of polylysine. There was a 66% increase in polylysine release if 10% MCPM was added compared to 0%. When 10% was compared with 5%, then there was 33% higher release of polylysine.

The amount released from all formulations was calculated in parts per million and a correlation could be made with the Minimum Inhibitory Concentration and viable counts experiments.

Polylysine is an effective antibacterial agent and its use in dental composites shows promising results in the experiments of this thesis. Based on this thesis results a concentration of between 2% and 5% of polylysine in the novel composite formulations would allow for inhibition of bacterial growth. Other studies have shown that adding these concentrations of polylysine to the formulations will not significantly affect chemical and mechanical properties (Panpisut *et al.*, 2016; Walters (a), 2016).

The novel composites discussed in this thesis have promising characteristics for minimally invasive tooth restorations.

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Appendix

Statistics: Univariate Analysis of Variance

Novel composite formulations with added polylysine

Figure 5-3 – Series 1 results

Composition	Compositio n	*p value* 6 hours	*p value* 24 hours
A1	A2	1.000	.000
	A3	1.000	.000
	A4	.084	.000
A2	A3	1.000	.097
	A4	.079	.028
A3	A4	.522	1.000

Figure 5-4 - Series 2 results

Composition	Composition	*p value* 6 hours	*p value* 24 hours
B3	B4	1.000	1.000
	B2	1.000	1.000
	B1	.002	.000
	B5	.578	.023
	Z250	.011	.000
B4	B2	1.000	1.000
	B1	.000	.000
	B5	1.000	.87
	Z250	.000	.000
B2	B1	.002	0.000
	B5	.576	.002
	Z250	.011	.000
B1	B2	.002	0.000
	B5	.000	0.000
	Z250	1.000	1.000
B5	Z250	.000	0.000

Figure 5-5 –

Series 3 Results (inoculum $5x10^6$ CFU/ml)

Composition	Composition	*p value*
C1	C2	.000
	C3	.000
	C4	.000
	C5	.000
	FUJI II	.064
	FUJI IX	.358
	inoculum	1.000
	Z250	.002
C2	C3	1.000
	C4	.269
	C5	.384
	FUJI II	.000
	FUJI IX	.000
	inoculum	.000
	Z250	.000
C3	C4	1.000
	C5	1.000
	FUJI II	.000
	FUJI IX	.000
	inoculum	.000
	Z250	.000
C4	C5	1.000
	FUJI II	.000
	FUJI IX	.000
	inoculum	.000
	Z250	.000
C5	FUJI II	.000
	FUJI IX	.000
	inoculum	.000
	Z250	.000
FUJI II	FUJI IX	.000
	inoculum	.003
	Z250	.000
FUJI IX	inoculum	1.000
	Z250	1.000
inoculum	Z250	.038

Figure 5-6 –

Series 3 results (inoculum $7x10^6$ CFU/ml)

Composition	Composition	*p value*
C1	C2	1.000
	C3	1.000
	C4	.122
	C5	.076
	FUJI II	1.000
	inoculum	1.000
	Z250	1.000
C2	C3	1.000
	C4	.072
	C5	.044
	FUJI II	1.000
	inoculum	1.000
	Z250	1.000
C3	C4	.752
	C5	.497
	FUJI II	1.000
	inoculum	.337
	Z250	1.000
C4	C5	1.000
	FUJI II	.092
	inoculum	.000
	Z250	.005
C5	FUJI II	.057
	inoculum	.000
	Z250	.003
FUJI II	inoculum	1.000
	Z250	1.000
inoculum	Z250	1.000

Figure 5-7 –
Series 4 Results (inoculum $8x10^5$ CFU/ml)

Composition	Composition	*p value*
control	D1	1.000
	D2	1.000
	D3	1.000
	D4	.104
	D5	.000
	D6	.000
	Z250	1.000
D1	D2	1.000
	D3	1.000
	D4	.059
	D5	.000
	D6	.000
	Z250	1.000
D2	D3	1.000
	D4	.491
	D5	.000
	D6	.000
	Z250	1.000
D3	D4	1.000
	D5	.000
	D6	.000
	Z250	1.000
D4	D5	.000
	D6	.000
	Z250	.416
D5	D6	1.000
	Z250	.000
D6	Z250	.000

Figure 5-8 -
Series 4 Results (inoculum $8x10^7$ CFU/ml)

Composition	Composition	*p value*
D1	D2	1.000
	D3	1.000
	D4	1.000
	D5	.000
	D6	.000
	inoculum	1.000
	Z250	1.000
D2	D3	1.000
	D4	1.000
	D5	.000
	D6	.000
	inoculum	1.000
	Z250	1.000
D3	D4	1.000
	D5	.000
	D6	.000
	inoculum	1.000
	Z250	1.000
D4	D5	.000
	D6	.000
	inoculum	1.000
	Z250	1.000
D5	D6	.000
	inoculum	.000
	Z250	.000
D6	inoculum	.000
	Z250	.000
inoculum	Z250	1.000

Mass and Volume

Figure 5-19 – Mass change

Composition	Composition	*p value*
10M 0.5P	10M 1P	.769
	10M 2P	.052
	5M 5P	.000
10M 1P	10M 2P	.707
	5M 5P	.001
10M 2P	5M 5P	.010

Figure 5-22 – Volume change

Composition	Composition	*p value*
10M 0.5P	10M 1P	1.000
	10M 2P	1.000
	5M 5P	1.000
10M 1P	10M 2P	1.000
	5M 5P	1.000
10M 2P	5M 5P	1.000

Polylysine release

Figure 5-24 –

Polylysine release percentage

Composition	Composition	*p value*
10M 0.5P	10M 1P	.115
	10M 2P	.915
	5M 5P	1.000
10M 1P	10M 2P	1.000
	5M 5P	.040
10M 2P	5M 5P	.306

Figure 5-26 -

Polylysine release milligrams

Composition	Composition	*p value*
10M 0.5P	10M 1P	.744
	10M 2P	.000
	5M 5P	.000
10M 1P	10M 2P	.001
	5M 5P	.000
10M 2P	5M 5P	.000

Figure 5-27 -

Polylysine release percentage

Composition	Composition	*p value*
10M 2P	5M 5P	.473
	0M 2P	.016
5M 5P	0M 2P	1.000

Audit of Inhalation Sedation practice in the paediatric department

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Abstract

Background: The Intercollegiate Advisory Committee for Sedation in Dentistry (IACSD) released new standards in 2015 for the provision of sedation. It is mandatory for records of the audit process and outcomes to be maintained and be available for inspection.

Aim: to evaluate the departments inhalation sedation record keeping.

Objectives:

- To establish how effectively the department uses the Inhalation Sedation (IS) logbook.
- To assess the outcomes of IS appointments.

Standard: 100% of treatments and outcomes recorded in the IS logbook

Methodology: A retrospective audit was done, with the first cycle in July 2015 and the second cycle in February 2016. Data was collected from the IS logbook and the appointment records system. 163 patients were assessed on the first cycle and 148 on the second.

Results:

- Logbook recording increased from 44% to 74%
- In both cycles 100% of outcomes was recorded
- Successful treatments remained similar at 93% and 94%
- In both cycles 42% of treatments were extractions and 38% restorations

Recommendations and action plan: The results were presented at departmental level. An email reminder was send to all staff. Laminated posters were placed in each IS room to remind clinicians to fill in the IS logbook.

Conclusion: This audit highlighted the need for accurate record keeping. Record keeping increased significantly in between cycles but did not achieve the gold standard. This will be reassessed via further audit cycle.

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Background

Introduction

The level of dental caries in children has dropped significantly over the last few decades. A substantial proportion however still has caries and a lot is left untreated. In the UK in 2013, a third of 5 years old and almost half of 8 year olds had previous decay in the primary dentition. Approximately a third of these children had received treatment (Pitts and Chadwick, 2013). In the USA almost 50% of 6 year olds had previous decay experience and 60% had received treatment (National Centre for Health Statistics, 1996). Having dental caries can have a detrimental effect on a person. It can lead to pain, swelling, loss of teeth and difficulty eating and drinking. There is evidence showing that invasive dentistry can create anxiety and young 5 year old children who have had pain/invasive dental treatment are more likely to be dentally anxious at the age of 10 (Oosterink et al., 2008; Raadal et al., 2002). This can lead to avoidance of dentists and further problems. This avoidance is usually be associated with high caries rate (Arnrup et al., 2003).

A large proportion of young children with caries are treated under general anaesthetic due to the load of treatment as well as being uncooperative. A general anaesthetic should however be avoided where possible due to a rare associated risk of death. Additionally, general anaesthetic is a costly procedure and requires specialist staff and facilities. The alternative is treatment under local anaesthesia, however this is not always accepted by children. Behaviour management as well as dental fear proved to be barriers to accepting dental treatment. Approximately 10.5% of children between 4 and 11 years old have dental fear and behaviour management problems (Klingberg *et al*., 1994).

In 1998 the General Dental Council (GDC), the regulatory body for dentistry in the UK, changed the guidelines for the provision of general anaesthesia for dental procedures. As a result, in July 2000 a document was published for the Department of Health (DoH) called *A Conscious Decision*. This was chaired by the Chief Medical and Chief Dental Officers. Following the release of the above document all general anaesthetic services for the provision of dentistry were only allowed to be done in a hospital setting. This changed the focus to the use of different methods for the control of pain and anxiety. This change resulted in a reduction in the use of general anaesthesia and an increase in the use of conscious sedation (Pike, 2000; Smith and Thompson, 2007).

Since the increase of the use of conscious sedation appropriate standards needed to be in place. In 2003 the UK standing Dental Advisory Committee published standards for

the use of sedation in dentistry with title *Conscious Sedation in the Provision of Dental Care*. This has been superseded a few times with the latest document published in 2015 by the Intercollegiate Advisory Committee for Sedation in Dentistry (IACSD) with title *Standards for Conscious Sedation in the Provision of Dental Care.*

These reports make recommendations for practitioners providing conscious sedation. It is important to consider alternative methods of pain and anxiety control, and also it is vital that theoretical, practical education and training is of high standard and is continuously updated. Additionally auditing the dental team as part of clinical governance to ensure high quality of care is required. Finally the team must maintain appropriate equipment and drugs and ensure these are well maintained (Pike, 2000; Farnfield, 2003). IACSD also recommended the ages at which sedation can be used. The main recommendation is a cut off at 12 years of age for the use of intravenous sedation. There is no minimum age for the use of inhalation sedation.

Behaviour management is divided into two categories: pharmacological and non-pharmacological. Non-pharmacological behaviour management (NPBM) involves techniques employed by dentists to reduce anxiety of patients, with the aim of completing treatment with the least distress possible. Common NPBM techniques include empathy, distraction, positive reinforcement and tell, show, do. These techniques when used effectively can shape the behaviour of a child and allow the treatment to be completed. However, many children may still find it difficult to tolerate dental treatment.

Pharmacological behaviour management is subdivided into two categories, general anaesthetic and conscious sedation, inhalation or intravenous. Different countries will have different definitions for what conscious sedation constitutes. This is because some sedation drugs are also used in different doses to induce general anaesthesia. The ideal sedative drug should reduce anxiety to the level that the patient allows treatment. It should be used safely in primary care and have a wide margin of safety. The drugs used in conscious sedation are benzodiazepines, mainly midazolam for intravenous/oral/rectal and nitrous oxide for inhalation.

According the general dental council and the UK department of health, conscious sedation is ‘’A technique in which the use of a drug or drugs produces a state of depression of the central nervous system enabling treatment to be carried out, but during which verbal contact with the patient is maintained throughout the period of sedation. The drugs and techniques used to provide conscious sedation for dental treatment should carry a margin of safety wide enough to render loss of consciousness unlikely’’. In the USA the definition of conscious sedation is very similar but the American academy

of Paediatrics also separates sedation to conscious and deep, where in deep sedation the patient is not easily aroused.

All children should be able to expect painless high-quality care. Although behaviour management may need to be used in conjunction with conscious sedation, pharmacological agents should not substitute effective communication and the management abilities of the operator. In 2002 the British society of paediatric dentistry released national guidelines for the use of conscious sedation in paediatric dentistry (Hosey, 2002). High risk sedative techniques such as polypharmacy are not indicated for the primary dental care, certainly in the UK, but also in parts of the world where deep sedation is more common. Inhalation sedation remains the preferred technique of pharmacological management for anxious paediatric patients. Restraining devices (such as the papoose board) and deep sedation techniques (where the patient is sedated more than what the definition of the GDC implies) are not acceptable in the UK. In the 2002 guidelines the indications and contraindications for inhalation sedation (IS) are outlined along with the evidence that supports it. Briefly, IS is indicated for children with mild to moderate anxiety to allow them to cope with multiple appointments and accept treatment but should not be used in isolation from the support given by the dentist. It can be used to facilitate extractions, and specifically it is indicated over a general anaesthetic for orthodontic premolar extractions. It is a weak analgesic and has minimal effect on the cardiovascular and respiratory systems. Contraindications are few and include first trimester of pregnancy, common cold, nasal blockage, bleomycin chemotherapy and pre-cooperative children (Hosey, 2002).

A Cochrane review in 2012 concluded that there is weak but consistent evidence that oral midazolam is effective in reducing anxiety compared to placebo with few minor adverse effects. Additionally there is weak evidence that inhalation sedation with nitrous oxide is more effective than placebo with no adverse effects (Lourenço-Matharu, Ashley and Furness, 2012). There is little evidence to show which age group is inhalation sedation most effective and appropriate. When prescribing drugs to children the British National Formulary (BNF) divides children in three groups, 1-6 years, 6-12 years and over 12 years of age. Based on the above, consideration should be given as to why sedation is provided. In pre-co-operative children (under 6) the intention is often to get the treatment done and the sedation may be the tipping point which enables the treatment to be done. In older children, for example over 12, the intention may be to make the treatment more pleasurable and as a result reducing anxiety for further visits. Additionally, sedation can aid significantly when more complex treatments are required.

The outcome of dental treatment has been shown to have an effect on how acceptable it is. Treatments that had positive outcomes have been rated as more acceptable compared to the ones with negative outcome (Newton, Naidu and Sturmey, 2003). Multiple studies have been previously completed about the acceptability of inhalation sedation and success rates range are good ranging from 83% to 100% (Crawford, 1990; *Shaw et al*, 1996; Bryan, 2002).

Nitrous oxide

Nitrous oxide is a colourless and odourless gas with a faint, sweet smell. It is an analgesic and anxiolytic agent which causes central nervous system depression and euphoria with negligible effect on the respiratory system. Nitrous oxide works in two different ways. The analgesic effect is introduced by release of endogenous opioid peptides. This is followed by activation of opioid receptors, descending Gamma-aminobutyric acid type A (GABAA) receptors and noradrenergic pathways that modulate processing at the spinal level (Emmanouil and Quock, 2007). Anxiolysis occurs by the activation of the GABAA receptor either directly or indirectly through the benzodiazepine binding site. Nitrous oxide is absorbed very quickly, which leads to quick uptake from the lung alveoli. Nitrous oxide is quite insoluble, and can pass down a gradient into other tissues and cells in the body. It is excreted quickly from the lungs. As nitrous oxide is 34 times more soluble than nitrogen in blood, diffusion hypoxia may happen. Children desaturate more rapidly than adolescents, and giving 100 percent oxygen to the patient once the nitrous oxide has been replaced is crucial (Patel *et al.*, 1994).

Nitrous oxide is absorbed quickly, allowing for a fast onset and recovery which can be as quick as two minutes. It causes negligible impairment of any reflexes, hence the cough reflex is protected (Paterson and Tahmassebi, 2003). It is very safe with no recorded deaths or cases of serious morbidity when used correctly (Nathan, 1989). Studies have concluded that negative outcomes may be linked with use of nitrous oxide in a greater than 50 percent concentration and as an anaesthetic during major surgery (Schmitt and Baum, 2008). Although uncommon, silent regurgitation and subsequent aspiration need to be taken into account with nitrous oxide/oxygen sedation. The problem lies as to whether pharyngeal-laryngeal reflexes remain intact. This issue can be avoided by ensuring the patient does not go into an unconscious state (Hogue, Ternisky and Iranpour, 1971).

Nitrous oxide has been linked with environmental concerns because of its role in the greenhouse effect. It is produced naturally by bacteria in soils and oceans. Humans produce it by burning fossil fuels and forests and through the agricultural practices of soil

cultivation and nitrogen fertilization. Nitrous oxide is responsible for about five percent of the greenhouse effect. Dental and medical practice contribute for less than two percent of this (Levering and Welie, 2011).

Adverse effects

Nitrous oxide is a safe drug to use to provide anxiolysis to young children. When used by trained dentists on patients that have been carefully selected and fall under the criteria discussed before, nitrous oxide is an effective and safe drug for providing pharmacological behaviour management in children. Adverse effects, acute or chronic, are rare (Donaldson and Meechan, 1995). Vomiting and nausea are considered the commonest side effects of inhalation sedation, occurring in about 0.5 percent of patients (Kupietz*ky et al.*, 2008). Fasting is not necessary for children having inhalation sedation. However it recommended that only a light meal is consumed in the two hours before the appointment (Hosey, 2002). Diffusion hypoxia can happen following rapid release of nitrous oxide from the blood stream into the alveoli, thereby diluting the concentration of oxygen. This can cause headaches and disorientation and can easily be avoided by administering 100 percent oxygen for 3-5 minutes after nitrous oxide has stopped (Paterson and Tahmassebi, 2003). For the dental team, it has been reported in the literature that long term exposure can be linked with bone marrow suppression and reproductive organ disturbances (Lehmberg *et al.*, 2008). Most of these reports were made when nitrous oxide was used as a general anaesthetic and active scavenging was not available. It is now recommended to minimise exposure by using effective scavenging and ensuring all equipment is maintained properly (AAPD Clinical Affairs Committee and AAPD Council on Clinical Affairs, 2009).

Sedation scoring

The patients level of consciousness during sedation and treatment can be assessed through their response to commands, verbal or physical. During dental procedures, the patient must be observed clinically at all times. The observations should include colour, respiratory rate and responsiveness. If a patient can speak, this indicates that they are breathing (American Society of Anesthesiologists Task Force on Sedation and Analgesia by Non-Anesthesiologists, 2002).

The airway becomes increasingly compromised and the likelihood of respiratory and cardiovascular depression increase as the depth of sedation progresses from light to deep. It is important that the dentist uses a universally accepted system to evaluate and record the level of sedation. Additionally, the accurate recoding of the sedation scoring is important from a medicolegal point of view. There are three widely used sedation

scoring systems, the Ramsay sedation scale (RSS), the modified Observers Assessment of Alertness/Sedation scale (OAA/S) and the Wilson sedation score, also known as the University of Michigan scoring system (UMSS). The RSS defines the conscious state from a level 1, where the patient is anxious, agitated or restless, through to a level 6, where the patient is completely unresponsive. The OAA/S is a six-point scale ranging from 5 to 0 that involves creating a response to an intense stimulus that begins with speaking with a normal voice to prodding and finally to a painful stimulus. Finally, the UMSS is a sedation scale that was originally developed for children but has also been adopted for use in adult sedation. It assesses level of consciousness objectively over five different points. The UMSS is an observational tool that scores the patients responsiveness to stimuli. This sedation scoring tool has been previously tested for inter-rater and test-retest reliability, and its validity in children of 6 months to 12 years is high, supporting its use in procedural sedation (Malviya *et al.*, 2002; McDermot *et al*., 2003). Furthermore the UMSS scores of 0-1 have been shown to be sensitive and specific in determining whether the patient has returned to baseline level of alertness and how ready he is to be discharged after sedation (Malviya *et al.*, 2004).

Apart from sedation scoring the dentist needs to assess the operating conditions. Depending on levels of anxiety, patient cooperation and level of sedation the conditions may be good, fair, poor or impossible. The patient may be fully cooperative with optimum degree of sedation, there may be minimal interference from the patient due to over/under sedation, the operation may be difficult due to over/under sedation and finally, the operation may be impossible. Operating conditions indicate whether a treatment has been successful, but not whether the sedation has been effective.

The final part of sedation record keeping is recovery. Recovery from inhalation sedation is rapid and only a few minutes of recovery need to be monitored. Recovery can be normal, within the timescale expected, rapid or prolonged. As part of record keeping, sedation scoring, operating conditions as well as recovery must be recorded and be available for inspection.

Audit guidelines

Any procedure that involves conscious sedation must be subject to regular audit and review. The members of the dental team should take part in this. This should involve the analysis of outcomes as well as the recording of procedures. All these documents will be part of a record and should be available for inspection.

Regular auditing of conscious sedation practice is essential and is considered to be a core requirement for those undertaking conscious sedation practice for patients (Dental

Faculties of the Royal College of Surgeons and the Royal College of Anaesthetists, 2015).

Conscious sedation procedures have to be the topic of regular audit in which all of the team must take part. Auditing should be an ongoing assessment of procedures and processes with review of outcomes and changes made to techniques and procedures as required. Records of the audit procedure and results from them must be kept and be accessible for review. Auditing of conscious sedation practice falls under the record keeping umbrella. Accurate record keeping in relation to dental care and inhalation sedation is necessary to ensure appropriate flow of treatment. It is also essential for medicolegal reasons. Information on the records is personal to a patient and is therefore confidential and encompassed by the Data Protection Act 1998 (Dental Faculties of the Royal College of Surgeons and the Royal College of Anaesthetists, 2015).

All sedation teams must have a clinical log which is be kept together with clinical records. Each clinical team must have contemporaneous and continuous records of the number and types of sedation treatments done together with the amount of any complications that may have happened (Dental Faculties of the Royal College of Surgeons and the Royal College of Anaesthetists, 2015).

Before the administration of nitrous oxide/oxygen, informed consent should be taken from the parent, ideally in a separate appointment, and recorded in the patient's record. The dentist should provide instructions to the parent regarding instructions before treatment if needed. Additionally, the patient's record should include the reason for use of nitrous oxide, the dosage used, including percentage of the mixture and flow, how long the procedure lasted, and recovery after treatment. (American Academy of Pediatric Dentistry, 2011).

Audit

Aim

In 2015 the new guidelines of the Intercollegiate Advisory Committee for Sedation in dentistry were released. It is mandatory that records of the audit process and outcomes from them must be maintained and be available for inspection. The aim of this audit was to evaluate the department's inhalation sedation record keeping. The objectives were to establish how effectively the department uses the inhalation sedation (IS) logbook and to assess the outcomes of IS appointments.

Standards

The standard was set to 100% as this was mainly a record keeping audit. 100% of treatment and outcomes should be recorded in the IS logbook and be available for inspection.

Methods and data source

The audit was carried out retrospectively. It was done in two cycles. The first cycle was in July 2015 and the second cycle in February 2016. The data was collected from three different sources. Initially all the appointments booked were recorded. The reception staff hold an Excel spreadsheet with the appointments booked. This is not updated with the daily changes to the appointments. However, the spreadsheet contains all the information regarding number of patients booked, date, time and patient details are recorded. From this spreadsheet, the initial number of patients booked was recorded. The second source of data was the outcome forms that were completed every time an appointment was completed. These forms are always completed and they include the treatment done. From the completed outcome forms information regarding type of treatment was also collected. The different types of treatment included restorations, restorations with preformed metal crowns, root canal treatment, extractions, surgical treatments, fissures sealants and other. These forms were not completed if the patient attended but did not have inhalation sedation in their appointment. As a result, by getting access to the outcome forms it was possible to find which patients attended and what treatment they had. Patients that attended but did not have IS treatment done had their status changed on the booking system to a normal appointment. Patients that did not attend or cancelled did not have an outcome form completed and did not have their appointment status changed to a normal appointment. Finally, the IS logbook was assessed and a comparison was made between which patients had an outcome form completed and which patients had been recorded in the IS logbook.

With the above method, it was possible to collect all the data regarding

- patients booked
- patients who attended and had IS appointment
- patients who attended but had a normal appointment
- patients who did not attend or cancelled
- number of patients who had IS appointment recorded in the IS logbook
- types of treatment completed

The remaining data was collected from the IS logbook. For the purposes of this audit only the outcome of the inhalation sedation appointment was recorded. Additional information in the logbook includes a patient identifier, type of sedation used, percentage of nitrous oxide and oxygen used, sedation scoring, recovery and signatures from the clinician and the dental nurse. In the cases were treatment had failed, the notes were retrieved to check what was the outcome of this appointment. Failed appointments would usually result in a new appointment for IS given, an appointment for use of intravenous sedation if the patient is over 12 or an appointment for a general anaesthetic if it was though that the patient wouldn't cope with another IS appointment.

All the data collected was inserted into an excel spreadsheet which included the patient identifier, date of appointment, whether it was completed or not and what treatment the patient had. Information on failed treatments was recorded manually as they were very few.

Below is an example of the excel spreadsheet used to record appointments and treatment completed.

Time	Hospital number	Patient name	Clinician	Treatment
09:00	41198300		PFAB7	NO IS
10:00	41196951		PFAC4	XLA
11:00	41032765		PFAB7	XLA
12:00	41085229		PFAC4	XLA
14:00	40996843		ALOAF	DNA
15:00	41191921		PFAB7	PMC
16:00				

Time	Hospital number	Patient name	Clinician	Treatment
09:00	41118627		PFAC1	CONS
10:00	02057161		PFAC1	CONS
11:00				
12:00	41153254		PFAB8	NO IS USED
14:00				
15:00	41074841		PFAC3	CONS
16:00	41097616		PFAC3	NO IS USED

Table 4: Example of data collection sheet

Below is the table used to record treatment in the IS logbook. for the purposes of this audit only the dental treatment and operating conditions were recorded.

Date	O, A, S, or S/D	Patient ID	Age Gender ASA	Sedation Technique Drugs/Doses	Dental Treatment	Comments: Sedation Scoring Operating Conditions Recovery

Table 5: Example of IS logbook layout

Results

The results of this audit were presented in the departmental monthly meeting as well as a poster in a national sedation meeting. The poster for the national meeting also included data of the same audit done for intravenous sedation.

First cycle

Below are the results of the first cycle which were recorded between the 22-06-2015 and 22-07-2015.

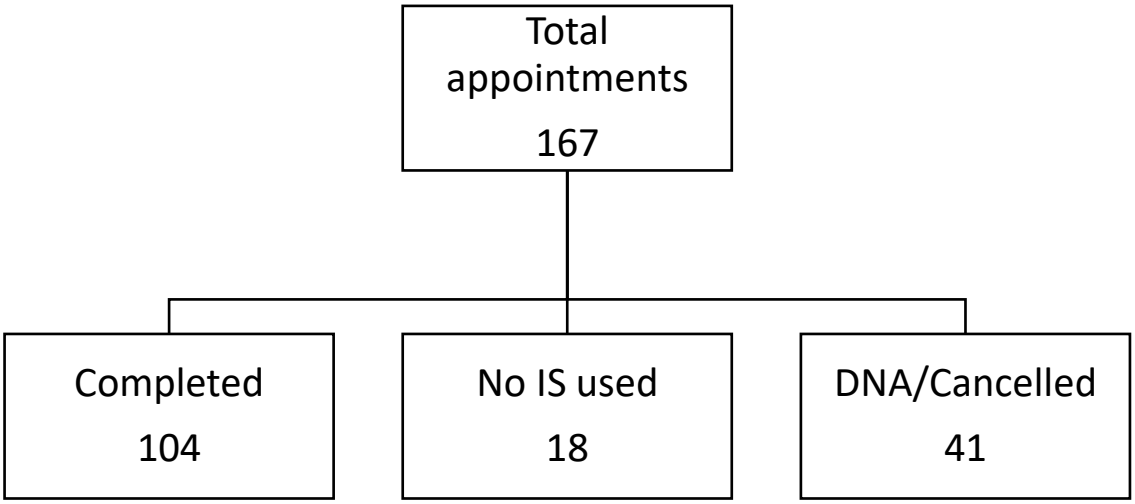

Figure 29: Booked and completed appointments – First cycle

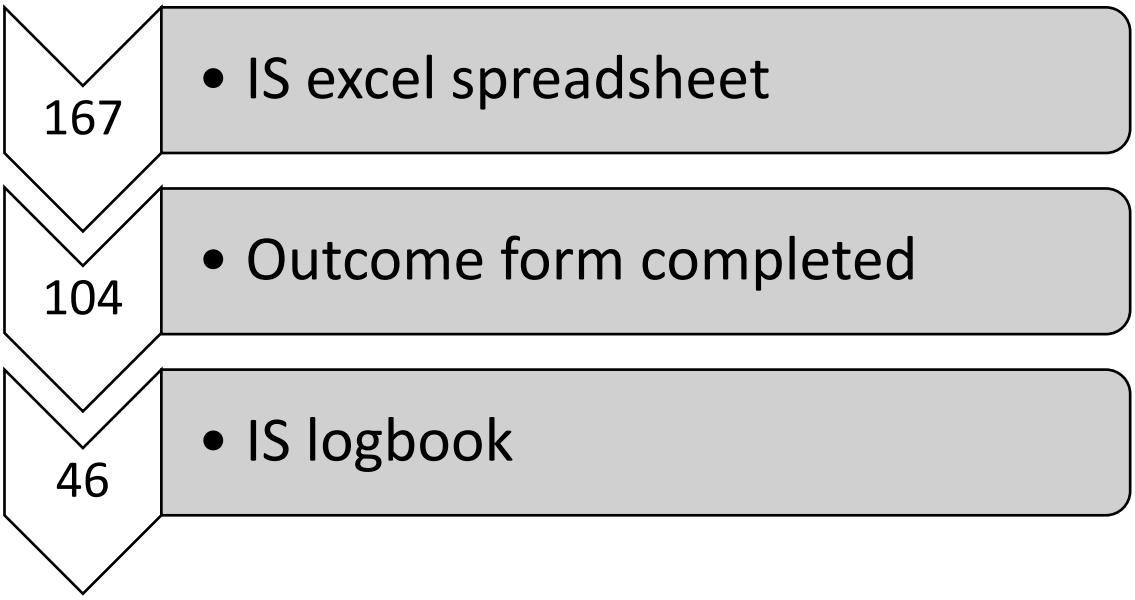

Figure 30: Booked, completed and recorded appointments – First cycle

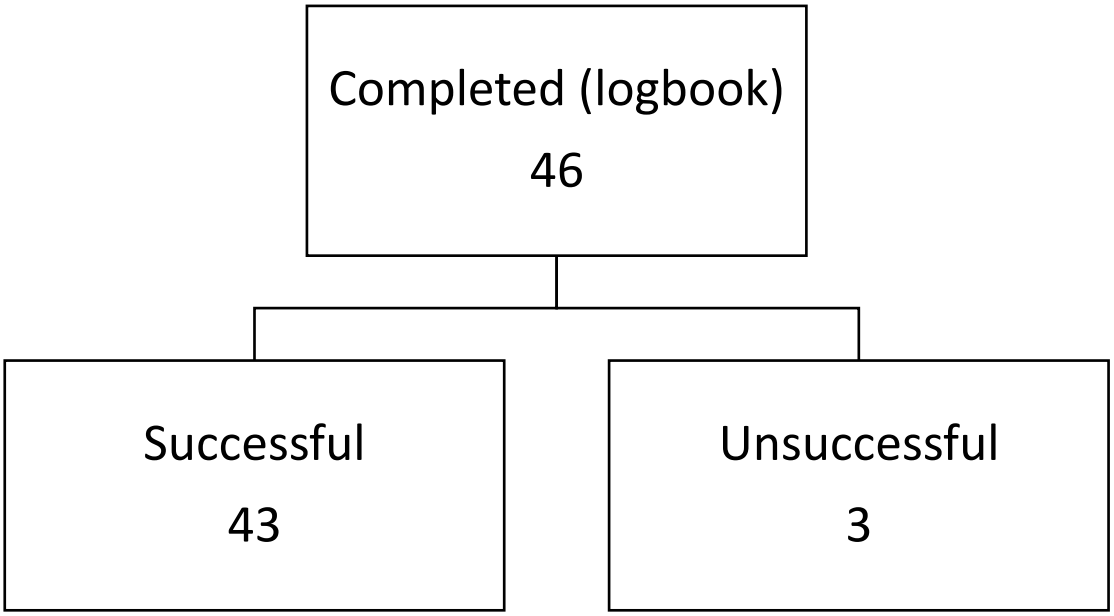

Figure 31: Successful appointments – First cycle

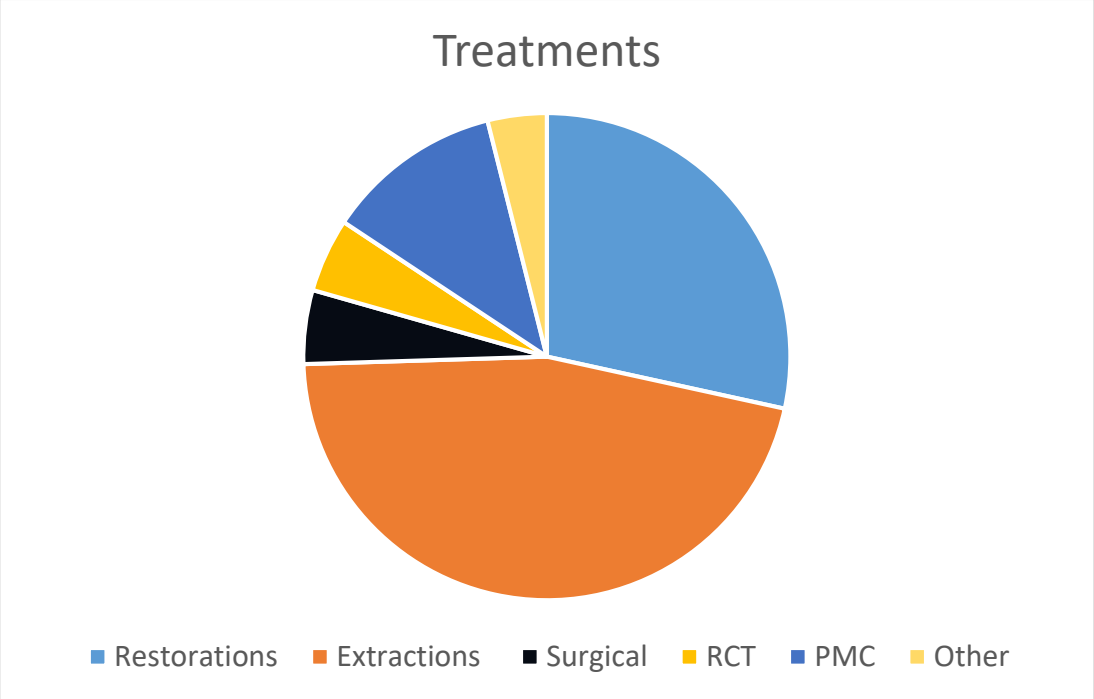

Figure 32: Breakdown of treatments – First cycle

	July 2015	
Total appointments	163	
Completed	104	64%
No IS used	18	11%
DNA/Cancel	41	25%
Logbook	46/104	44%
Successful	43/46	93%

Table 6: Result from first cycle – First cycle

At the end of the first cycle there were two major points which would require attention. Firstly the high ‘did not attend’ rate which was 25% and secondly the poor record keeping with only 44% of appointments recorded in the IS logbook.

Recommendations

The results were presented in the monthly departmental meeting and recommendations were made regarding the poor record keeping. These were to:

- circulate an email to all staff to ensure the logbook is been always completed
- place laminated posters in the two IS rooms to remind staff to complete the logbook

Following the above a plan was made to repeat the audit approximately six months later.

Second cycle

Below are the results of the second cycle which were recorded between the 01-02-2016 and 30-02-2016.

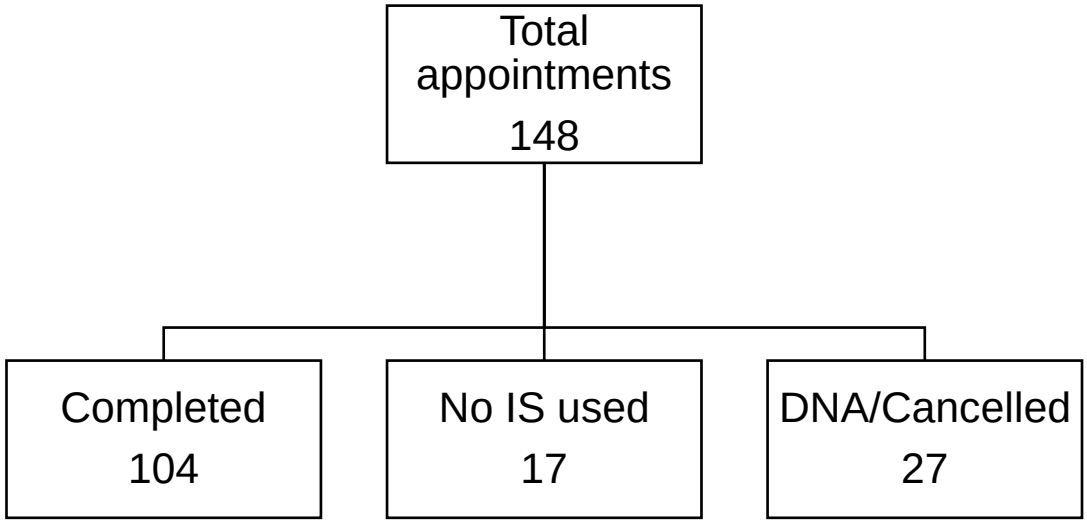

Figure 33: Booked and completed appointments – Second cycle

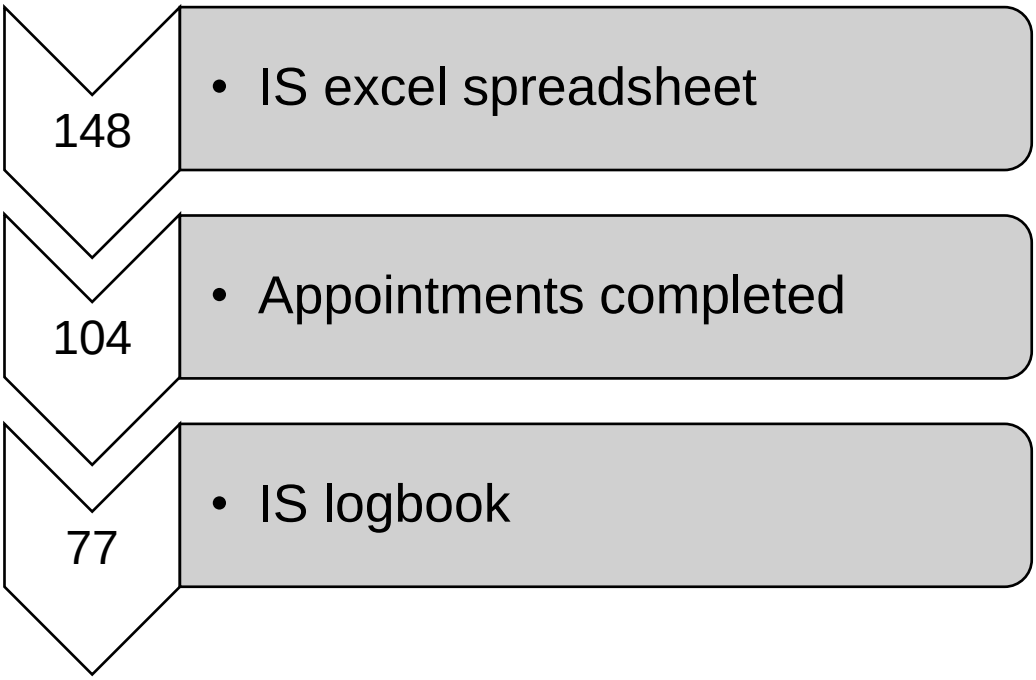

Figure 34: Booked, completed and recorded appointments – Second cycle

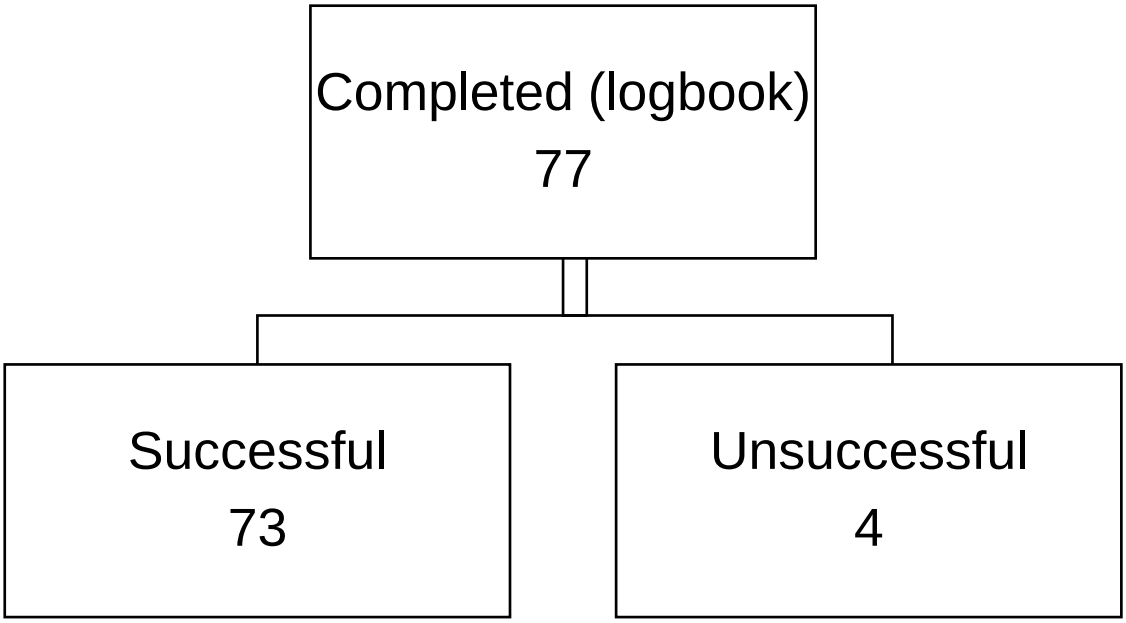

Figure 35: Successful appointments – Second cycle

	July 2015		February 2016	
Total appointments	163		148	
Completed	104	64%	104	71%
No IS used	18	11%	17	11%
DNA/Cancel	41	25%	27	18%
Logbook	46/104	44%	77/104	74%
Successful	43/46	93%	73/77	94%

Table 7: Result from both cycles

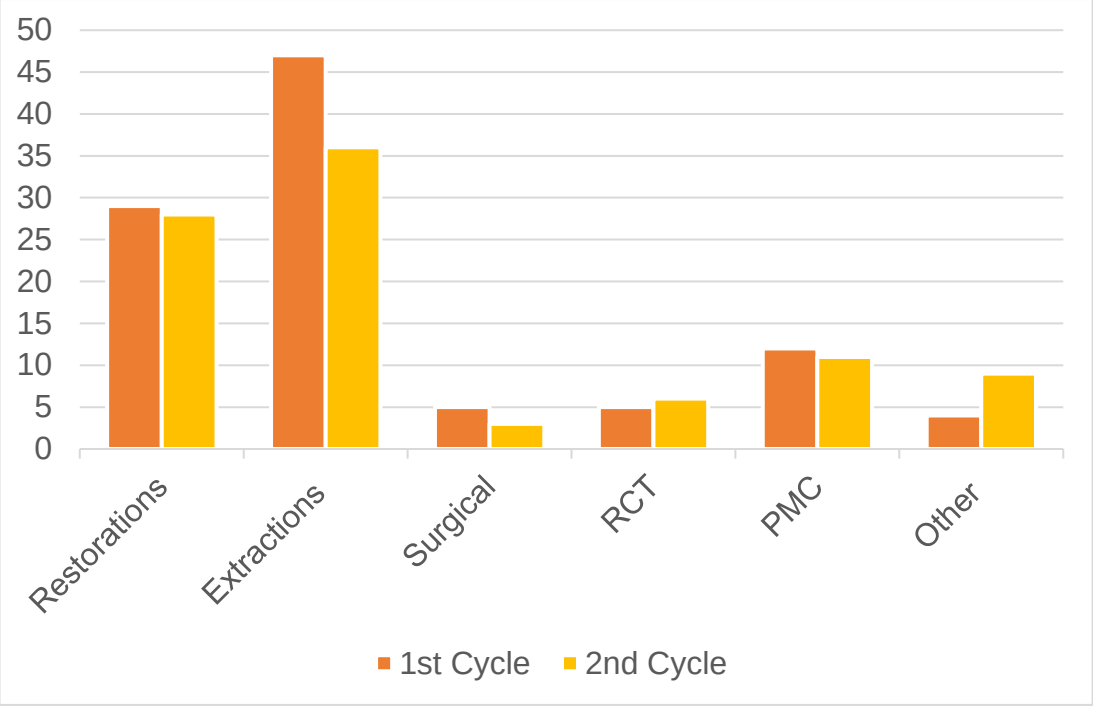

Figure 36: Breakdown of treatments - Both cycles

The second cycle was completed in February 2016. Further to the recommendations after the first cycle a significant improvement in record keeping was noted. This increased from 44% to 74%. The diversity of treatments completed remained similar with restorations (including PMC) and extractions accounting for approximately 78% of all treatments provided. There were eight failed treatments in the two cycles. Out of these four were failed extractions and in the other four the patients wouldn't allow treatment to commence. Four of these patients went on to have a general anaesthetic, three had treatment under IV sedation and one patient reattended for further treatment under IS.

Discussion

Inhalation sedation is used routinely in the department of paediatric dentistry as seen by the high numbers of appointments in the two cycles of the audit where approximately 155 appointments per month are booked. It is important that records of these appointments are appropriately kept and that these are audited regularly. The reason for that is to ensure a high standard of record keeping is maintained and also a high standard of inhalation sedation service is provided.

The departments high success rate of 95% is in accordance with previous research as discussed before where success rates can be between 83% and 100%. This is however one of the limitations of this audit, where failed treatment was recorded, not failed inhalation sedation. For example, an extraction could have been completed deeming the treatment successful but that does not necessarily mean that the patient was adequately sedated. It is crucial that in future audits both the outcome of treatment and outcome of sedation is recorded, as it is likely that successful sedation based on the sedation scoring will be lower than successful treatments. All the failed appointments were either failed extractions or appointments were although sedation commenced treatment did not commence at all.

The ''did not attend'' rate of 25% in the first cycle can be considered high. Although not part of this audit a telephone service was introduced between the audit cycles to remind patients of all sedation appointments. The results of this were significant and the DNAs were reduced from 25% to 18% in only a few months.

As expected the breakdown of different treatments remained the same over the two cycles, with the majority (>1/3) of treatments completed under inhalation sedation being dental extractions. Dental extractions are regarded amongst the most stressful procedures, especially for young children and as a results sedation is often offered for these cases.

One of the biggest improvements in between the two cycles (which was also the reason for this audit) is the significant increase in recording of sedation appointments in the logbook. This was initially very low at 44%. When an entry was made in the logbook, the outcome was always recorded too, hence recording of outcomes was 100% for both cycles. Because logbook recording was so low, it was not possible to fully assess success rates and complications in the first cycle as only 44% of the records was available for inspection. There are multiple reasons why record keeping was so low. Staff change continuously in the department and training is only limited to a few days. New staff may not be aware of sedation logbooks until after a few weeks into the job. A suggestion for this aspect is for dental nurses to also be responsible for the logbook. Dental nurses trained for sedation are usually more senior and do not change position often. On the other hand, new dentists come in the department every few months, many without any prior inhalation sedation experience. Record keeping advice should be part of the induction of new dentists.

After the first cycle when the poor record keeping was noted, actions were taken, mainly an email reminder, a staff meeting presentation and posters in both sedation rooms. These actions were enough to ensure all staff remembered to put an entry in the logbook at the end of each sedation appointment. Record keeping increased to 74%. This not only enabled compliance with record keeping standards set out by the General Dental Council but also ensured that records can be accessible for auditing.

As part of this audit the outcomes of sedation were not recorded apart from operating conditions. Future audits should also comment on sedation scoring and recovery, the two aspects that were not included in this audit. Additionally, the percentage of nitrous oxide given should be recorded as well as the American Society of Anaesthesiologists score.

Conclusion

This audit highlighted the need for contemporaneous and complete record keeping for inhalation sedation appointments. Record keeping was below standard at the end of the first cycle and this improved significantly following the multiple actions taken to address that. After the second cycle record keeping was still not perfect and this warrants the need for further auditing as well as regular reminders to all staff to ensure record keeping is of high standard in the department of paediatric dentistry.

The results of this audit, together with results of the same audit for intravenous sedation, were presented at national level in the UK, which ensured that the findings and recommendations were shared amongst peers.

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Case Number 1

Caries Case

Nikolaos Lygidakis

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2017

CASE SUMMARY

AJ is a fit and well young girl referred by her GDP regarding dental caries affecting her primary dentition. She has no previous pain from her teeth.
She presented with caries in 6 of her primary teeth. AJ was introduced to dental treatment gradually. A combination of composite fillings, conventional crowns, extraction, as well as fissure sealants and fluoride varnish application was carried out. Treatment was carried out using non pharmacological behaviour management and inhalation sedation.
Cooperation was difficult in the beginning but improved over time.
The treatment was successful.

PATIENT DETAILS

Initials: A.J.

Gender: Female

Age at start of treatment: 5 years and 10 months

Age at last review: 7 years

PRE-TREATMENT ASSESSMENT

HISTORY OF PRESENTING PATIENT'S COMPLAINT

- Mother and dentist noticed caries on multiple teeth.
- No history of pain, swelling or use of antibiotics.

RELEVANT MEDICAL HISTORY

- Delivered full term.
- No known allergies, no medical conditions and up to date vaccinations.

DENTAL HISTORY

- Two previous visits but never had any treatment.

FAMILY HISTORY

- None.

SOCIAL HISTORY

- Lives with parents and six siblings.
- Year one at school. Attends with her mother.

DIET

- Breast fed until 12 months old. Bottle fed until 2 years old.
- Has fruit juice and biscuits regularly.

ORAL HYGIENE

- Brushes once daily with adult toothpaste and manual toothbrush and rinses afterwards.

HABITS

- None.

CLINICAL EXAMINATION

EXTRA-ORAL EXAMINATION

- No facial asymmetry
- No lymphadenopathy
- No TMJ abnormalities
- Normal height/weight
- Very shy, allowed examination

INTRA-ORAL EXAMINATION

- Soft tissues – healthy appearance
- Oral hygiene – fair with some plaque deposits present
    - Simplified plaque index – 28% (OHI-S) (Greene and Vermillion 1964)
- Hard tissues
    - Teeth present

E	D	C	B	A	A	B	C	D	E
E	D	C	B	1	1	B	C	D	E

    - Carious teeth

E									E
E		D						D	E

- Occlusion
    - Class I deciduous molar occlusion
    - Early mixed dentition
    - Anterior openbite
    - Left side buccal segment crossbite

PRE-TREATMENT PHOTOGRAPHS

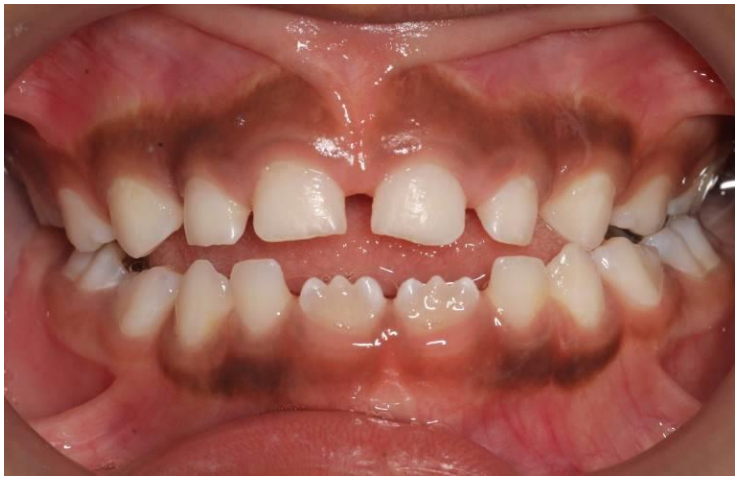


Figure 1: Anterior view (26-1-2015)

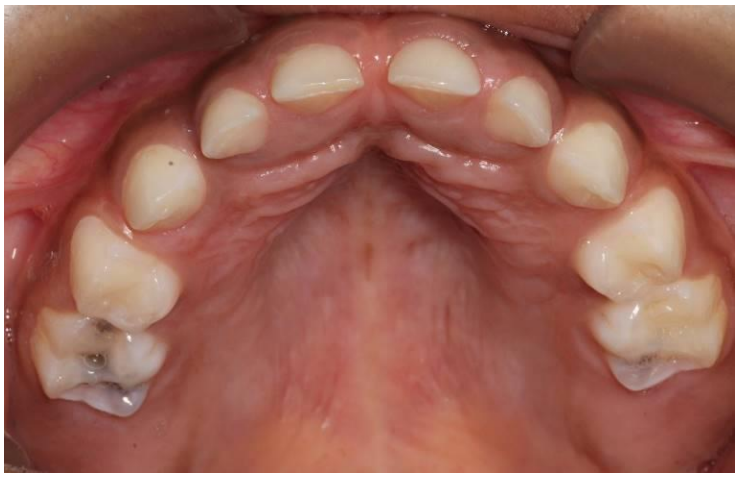


Figure 2: Maxillary occlusal view (26-1-2015)

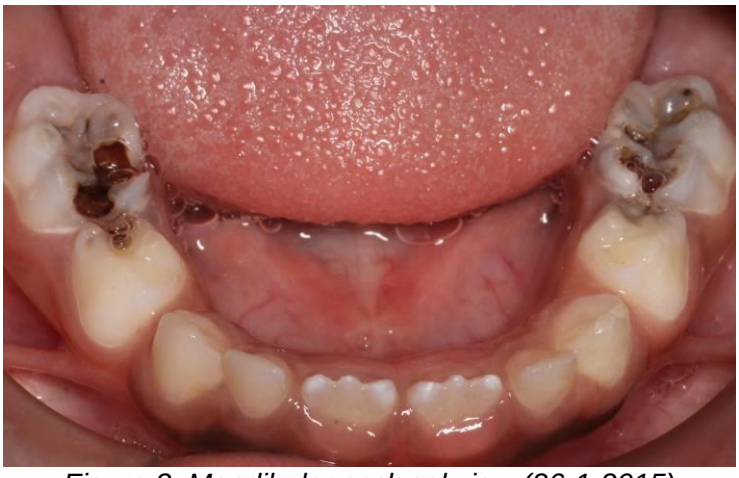
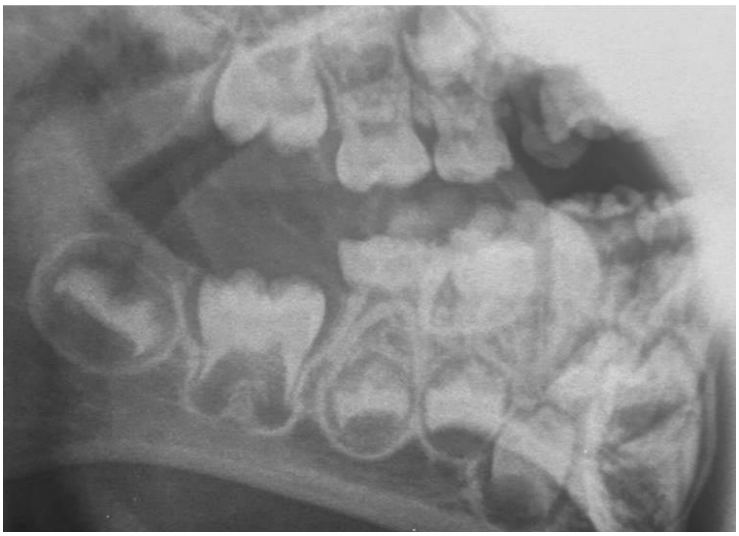


Figure 3: Mandibular occlusal view (26-1-2015)

GENERAL RADIOGRAPHIC EXAMINATION

Radiographs taken

- Right and left bimolars
- Justification - caries



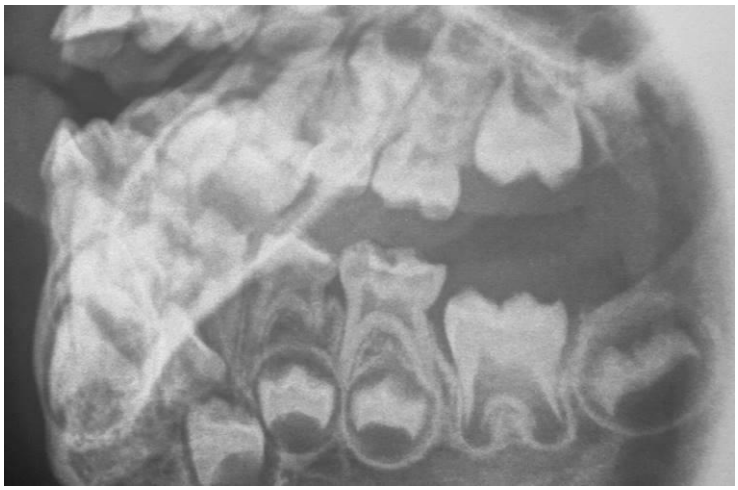


Figure 4 and 5: Bimolar radiographs (26-1-2015)

Radiographic findings

- Periapical radiolucency associated with LLD
- Caries see on LLE, LLD, URE, LRE
- Permanent premolars and molars present

DIAGNOSTIC SUMMARY

- Poor oral hygiene
- Dental caries affecting six primary teeth secondary to diet
- Periapical pathology LLD
- Dental anxiety

AIMS AND OBJECTIVES OF TREATMENT

- Improve oral hygiene through instructions, fluoride advice, and dietary education
- Restore oral health
- Manage anxiety and promote positive attitude towards dentistry
- Monitor development of permanent dentition

TREATMENT PLAN

1. Acclimatisation and prevention
    - Establish a preventive regimen consistent with the Department of Health preventive toolkit
    - Use of non-pharmacological behaviour management (NPBM) and inhalation sedation as a pharmacological behaviour management technique

2. Quadrant dentistry
    - Maxillary right quadrant
        i. URE composite restoration
    - Maxillary left quadrant
        i. ULE composite restoration
    - Mandibular right quadrant
        i. LLE PMC
        ii. LLD extraction
    - Mandibular left quadrant
        i. LRE PMC
        ii. LRD PMC

3. Fissure sealant of all first permanent molars once erupted

4. Maintenance and follow up
    - Monitor restorations
    - Radiographic review
    - Monitor development of dentition

TABLE OF MATERIALS USED

	Material used	Description
A	Topical Anaesthesia	Topical Anaesthetic Gel/Liquid – 20% Benzocaine, Dentsply, Milford, DE, USA
B	Local Anaesthesia (LA)	Lignocaine Special, 2.2 ml (2% Lidocaine, 1 :80000 epinephrine); Septodont, Saint –Maur-des-Fosses, France
C	Composite Material	Filtek Supreme XTE, 3M Espe, USA
D	Fissure sealant	Fissure Sealant Delton; Dentsply, Australia
E	Acid etch	Phosphoric Acid Gel Etchant 37.5%; Kerr Corporation, USA
F	Bonding agent	Opti bond Solo plus, single component, Total etch bonding agent, Kerr Co., California, USA
G	Preformed metal crowns (PMCs)	Stainless steel primary crowns; 3MTM ESPETM, St Paul, Minn, USA
H	Glass Ionomer Luting cement	Aquacem®; Dentsply, Milford, DE, USA
I	Duraphat®	Colgate Duraphat Varnish 50mg/ml dental suspension; Colgate Oral Pharmaceuticals, New York City, USA

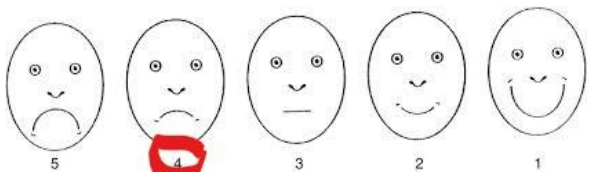
TREATMENT UNDERTAKEN

Visit 1 (26-1-15)

- Patient attended all the appointments with her mother
- Complete history taken
- Clinical and radiographic examination
- Clinical photographs
- Treatment plan formulated
- Discussion about Inhalation Sedation (IS) and leaflet given
- Written consent for IS obtained
- Oral hygiene instruction (OHI) given according to the DoH toolkit for prevention [1]
- Plaque score: 27% (OHI-S)

Visit 2 (31-3-15)

- No signs and symptoms, oral hygiene reinforced
- URE
    - Inhalation sedation (30% Nitrous oxide and 70% Oxygen, titrated)
    - Topical anaesthetic and 1.8ml 2% lidocaine with adrenaline 1/80000
    - Rubber dam isolation
    - URE occlusal caries removed, 3 cavities
    - Restored with A1 composite
    - 100% Oxygen for 5 minutes and post-operative instructions given (POIG)
- Patient was moving a lot during procedure and needed positive verbal instructions
- Duraphat® fluoride varnish 22600ppm applied to all teeth
- Treasure card given
- Behaviour:



Visit 3 (28-4-15)

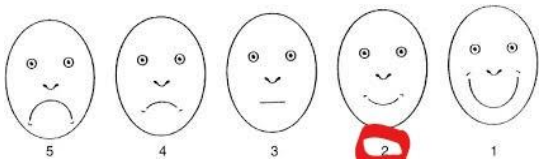
- No signs or symptoms, improved oral hygiene
- ULE
    - Inhalation sedation, topical and local anaesthesia as described before
    - Rubber dam isolation
    - Occlusal caries removed ULE
    - Restored ULE composite A1
    - POIG
- Needed verbal instructions and had limited attention span

Visit 4 (5-5-15)

- No signs or symptoms
- LRD, E
    - Inhalation sedation, topical and local anaesthesia as described before
    - LRD,E proximal and occlusal preparation done for Preformed metal crown (PMC)
    - Partial caries removal on LRE, distal caries on LRD removed during preparation
    - Cemented LRD size 4 and LRE size 5 PMC with Aquacem®
    - Good occlusion
    - POIG
- Cooperation improved
- Treasure card updated

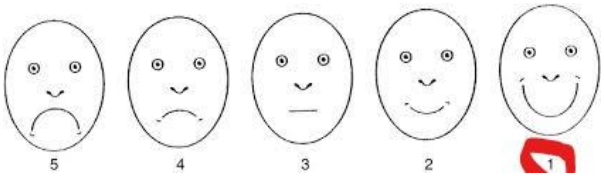
Visit 5 (16-6-15)

- No signs or symptoms, Oral hygiene improved
- LLD, E
    - Inhalation sedation, topical and local anaesthesia as described before
    - LLE proximal and occlusal preparation done for PMC
    - Partial caries removal on LLE
    - Cemented LLE size 5 PMC with Aquacem®
    - Extracted LLD intact using elevators and forceps
    - Good occlusion
    - Haemostasis
    - POIG
- Good cooperation today
- Duraphat® fluoride varnish 22600ppm applied to all teeth
- Treasure given
- Behaviour:



Visit 6 27-1-16

- No signs or symptoms, Oral hygiene improved
- Plaque score: 11% (OHI-S)
- New bitewings and clinical photos done today
- Duraphat® fluoride varnish 22600ppm applied to all teeth
- Fissure sealants done on all first molars
- Behaviour:



POST-TREATMENT PHOTOGRAPHS

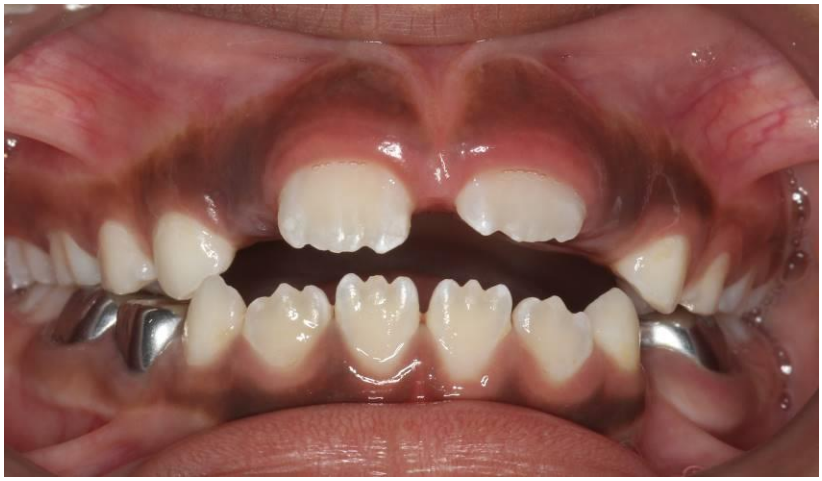


Figure 6: Anterior view 27-1-2016

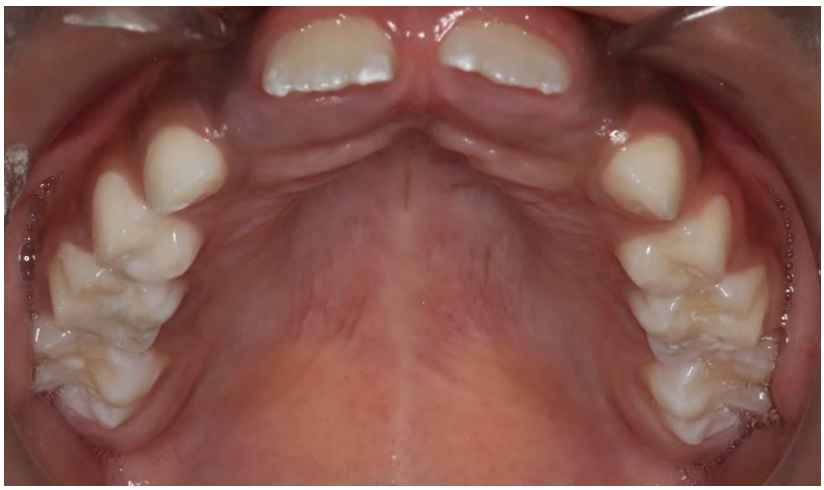


Figure 7: Upper occlusal view 27-1-2016

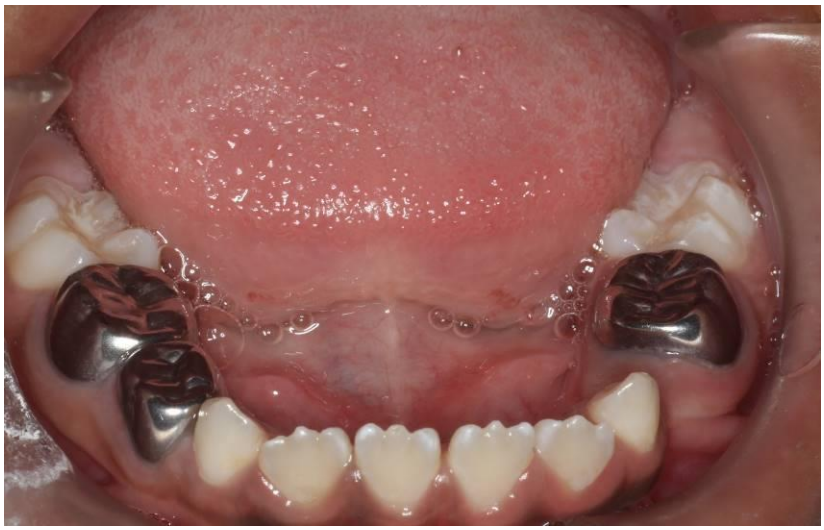


Figure 8: Lower occlusal view 27-1-2016

POST-TREATMENT RADIOGRAPHS

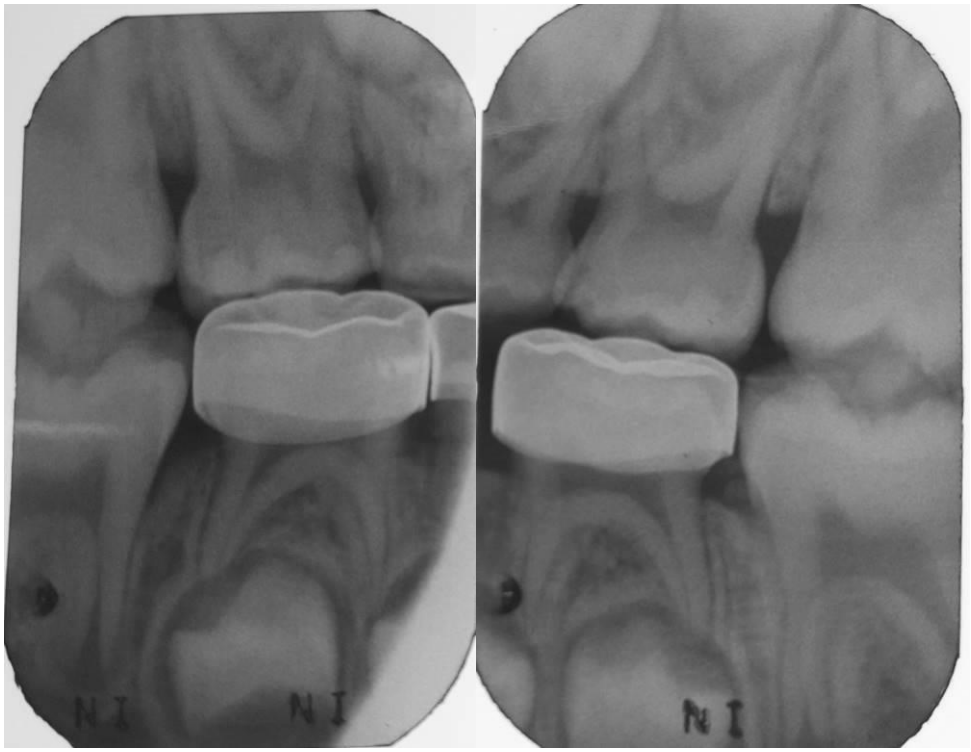


Figure 9: Vertical bitewings (27-1-2016)

Radiographic findings

- No recurrent caries
- No signs of periapical pathology

LONG TERM TREATMENT PLAN AND FUTURE CONSIDERATIONS

AJ has been discharged back to her GDP who will continue to offer prevention and apply fluoride varnish every three months.

DISCUSSION AND REFLECTION ABOUT CASE PRESENTED

The case was overall successful. It can be difficult to manage a young child who presents with caries affecting multiple teeth and when they have had no previous exposure to dentistry. Treatment has to happen in stages, efficiently and effectively and aim to maintain cooperation. In this case the treatment was completed chairside and a general anaesthetic was avoided. This not only ensured the treatment was carried out safely but also by completing the treatment a positive attitude towards dentistry was achieved.

Prevention

Prevention was given according to the 'Delivering better oral health: an evidence based toolkit for prevention' from the Department of Health (3rd edition)[1]. The mother was advised to use a toothpaste with fluoride concentration of 1450ppm and brush twice daily with spiting and not rinsing. A pea size amount of toothpaste should be used. Tooth brushing instructions were given using the modified bass technique. Diet advice was given with the aim of reducing sugar attacks during the day. Finally, as fluoride varnish of 22600ppm should be applied four times per year, a recall interval was set to 3 months as AJ is high caries risk[2]. AJ's oral hygiene improved over the course of treatment. On the second visit a simplified plaque index was done and oral hygiene was poor with a score of 27% (OHI-S, Greene and Vermillion, 1964) and in the last visit 11% so a marked improvement but not perfect.

Behaviour management

Inhalation sedation (IS) was used as a pharmacological behaviour management with AJ. A combination of IS and non-pharmacological behaviour management techniques made the treatment possible. IS has a favourable outcome when treating children with mild to moderate anxiety[3]. Tell show do was used effectively and AJ was interested to know how everything worked before treatment started. We managed to complete all the treatment without losing cooperation and promoted a good attitude towards dentistry for the future. The facial image scale was used to assess the anxiety of AJ. Although she was quite nervous for the first visit, this anxiety reduced over the course of the treatment and at the last appointment she was much more relaxed. The facial image scale is a validated system of assessing anxiety[4].

Treatment

Considering the sequence of treatment, when AJ first presented she had a periapical pathology present on the radiograph of the LLD. However, she was not in any pain. At the time AJ was 5 years old and had never had treatment before. The decision was made to postpone the extraction towards the end of the treatment as there was a chance of losing cooperation by attempting the extraction at the beginning. If there was pain on presentation the treatment plan would have been different. The mother was happy that if the LLD became symptomatic we would plan to extract it straight away.

We decided to use conventional crowns instead of the Hall technique due to the extent of caries on the lower second primary molars. We knew that although the cavities were large on the lower second molars there was no pathology radiographically and there were no signs of infection clinically. Partial caries removal was done before sealing the cavities with preformed metal crowns. The same happened for both second molars but not for the LRD which had a smaller distal cavity. This was only prepared mesially and distally to create space as both crowns on the lower right quadrant were cemented on the same day. Preformed metal crowns with conventional preparation have an excellent prognosis with success rates of 97% after 5 years[5]. Similarly Hall crowns have a success rate of 97% after 5 years[6].

Overall this was a successful case made possible using behaviour management techniques and gradual introduction to treatment.

LESSONS LEARNED

- Prevention advice and its importance
- Value of conservative management of caries

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6. Innes NPT, Evans DJP. Modern approaches to caries management of the primary dentition. *Br Dent J*. 2013;214(11):559-566.

Case Number 2

Trauma Case

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2017

CASE SUMMARY

AH is a 10 year old fit and well boy. He sustained trauma to the maxillary central incisors while playing Karate in April 2015. This resulted in enamel dentine pulp fracture of the maxillary central incisors.

AH presented five months after the initial injury for assessment. He was a very anxious young boy. The maxillary central incisors were built up with direct composite and root canal treatment was completed on both maxillary central incisors. Inside outside bleaching was undertaken.

Treatment was completed using non pharmacological behaviour management and inhalation sedation.

PATIENT DETAILS

Initials: AH

Gender: Male

Age at start of treatment: 10 years old

Age at last review: 11 years old

PRE-TREATMENT ASSESSMENT

HISTORY OF PRESENTING PATIENT'S COMPLAINT

- History of trauma
    - The maxillary incisors were fractured during a karate lesson. AH was accidentally elbowed. The injury was witnessed. There was no loss of consciousness.
    - The patient went to A&E and was cleared of head injury.
    - Subsequently the dentist extirpated the maxillary central incisors and placed temporary restorations.
    - No history of antibiotic use or swelling associated with the maxillary incisors.

RELEVANT MEDICAL HISTORY

- Fit and well with no known allergies.

DENTAL HISTORY

- Regular attender and after the accident attended the GDP a few times.
- Previously had local anaesthetic for the extirpation of the maxillary incisors.
- Very bad experience which left him very nervous of dental treatment.

SOCIAL HISTORY

- AH is in year 5 at school and has one sibling. He leaves with his parents.

DIET

- Diet is low in sugar. He likes to snack on olives and mainly drinks water.

ORAL HYGIENE

- AH brushes twice daily with a manual toothbrush using fluoridated toothpaste.

HABITS

- None.

CLINICAL EXAMINATION

EXTRA ORAL EXAMINATION

- No facial asymmetry
- No lymphadenopathy
- No TMJ abnormalities

INTRA ORAL EXAMINATION

- Soft tissues – healthy appearance
- Oral hygiene – good with minimal plaque deposits present.
    - Simplified plaque index – 11% (OHI-S) (Greene and Vermillion 1964)
    - BPE -

0	1	0
0	0	0

- Hard tissues
    - Mixed dentition
    - Teeth present

6	E	4		2	1	1	2	C	4	5	6
6	E	4		2	1	1	2		D	E	6

    - Enamel Dentine Pulp (EDP) fracture of UR1, UL1
    - UR1 GIC and UL1 Zinc Oxide-Eugenol temporary restorations
    - No abnormal mobility
    - Maxillary canines palpable
- Occlusion
    - Class I molar relationship
    - Mandibular midline shift to the right
    - 4mm Overjet and 3mm Overbite

- Trauma assessment

Test	*UR2*	*UR1*	*UL1*	*UL2*
EPT	58	-	41	41
Ethyl Chloride	+ve	-ve	-ve	+ve
TTP	-	-	-	-
Colour	-	discoloured	-	-
Sinus	-	-	-	-

Table 1: Vitality assessment

PRE-TREATMENT PHOTOGRAPHS

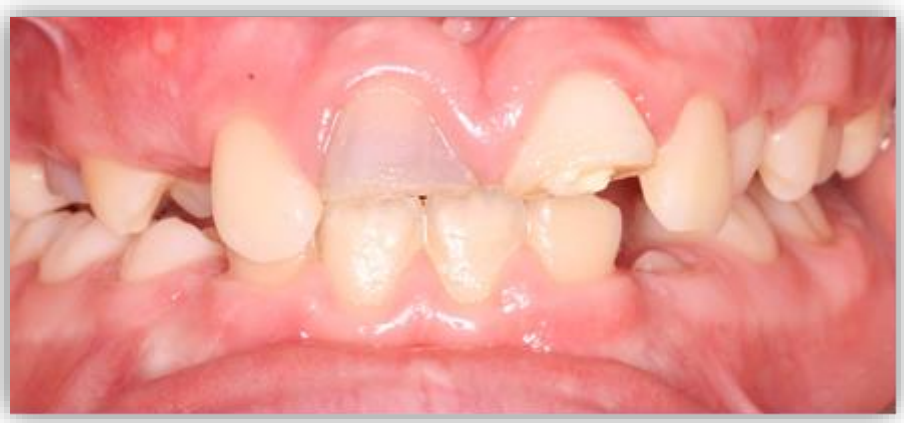

Figure 5: Anterior view (22-9-2015)

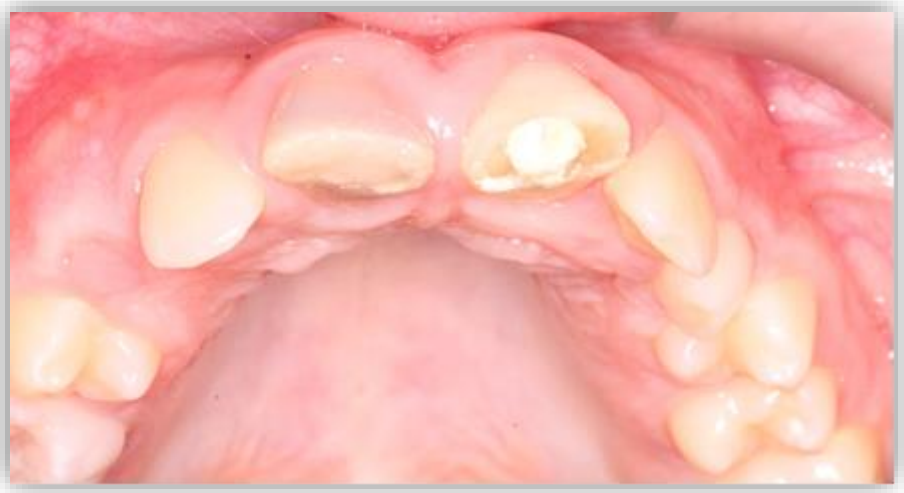

Figure 6: Maxillary occlusal view (22-9-2015)

GENERAL RADIOGRAPHIC EXAMINATION

Radiographs taken

- Long Cone Periapical radiograph of maxillary central incisors
- Assess for root fracture and periapical pathology.

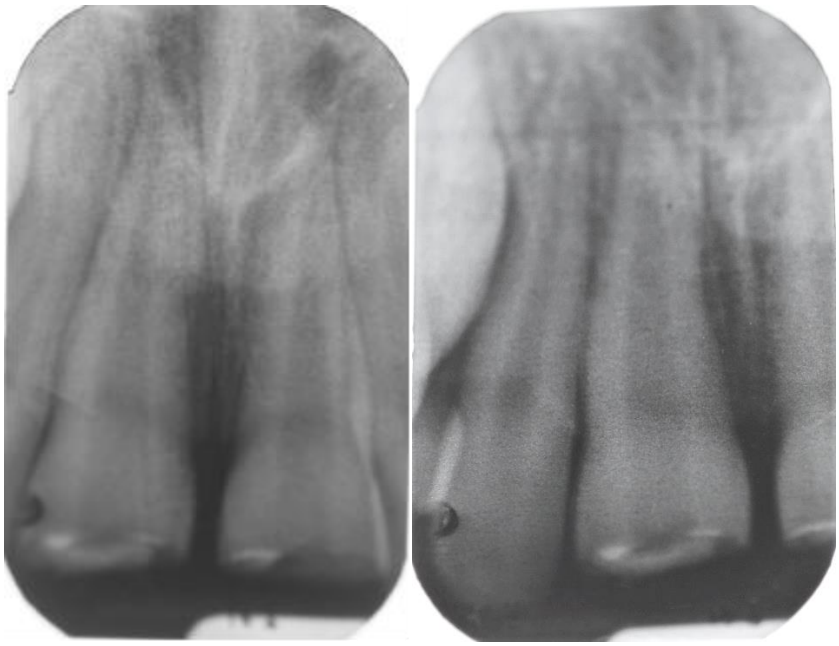

Figure 7: Periapical radiographs of maxillary incisors (22-9-2015)

Radiographic findings

- Periapical radiolucencies associated with the UR1, UL1
- Enamel Dentine Pulp fractures with restorations on UR1, UL1
- UR1, UL1 have almost closed apices
- No evidence of any endodontic medicament

DIAGNOSTIC SUMMARY

- Enamel Dentine Pulp fracture of maxillary central incisors leading to loss of vitality
- Dental anxiety

AIMS AND OBJECTIVES OF TREATMENT

- Manage anxiety and promote positive attitude towards dental treatment
- Maxillary central incisors
    - Complete endodontic treatment
    - Restore with direct composite
    - Non vital inside outside bleaching
- Monitor development of permanent dentition

TREATMENT PLAN

- Behaviour management
    - Due to AH's anxiety it was decided treatment should be completed under inhalation sedation

- Initial treatment
    - Dress root canals of the maxillary central incisors
    - Composite build-up of maxillary central incisors
    - Prevention according to the Department of Health Toolkit

- Long term treatment
    - Obturation of maxillary central incisors
    - Inside outside bleaching

- Follow up and maintenance
    - Clinical review every 6 months and radiographic review every 6-12
    - months
    - Reinforce OHI
    - Monitor restorations

TABLE OF MATERIALS USED

	Material used	Description
A	Electric pulp test	Kerr; Analytic Technology Corp, Redmond, WA
B	Ethyl chloride test	Endo cold spray, Henry Schein, UK
C	Topical Anaesthesia	Topical Anaesthetic Gel/Liquid – 20% Benzocaine, Dentsply, Milford, DE, USA
D	Local Anaesthesia (LA)	Lignocaine Special, 2.2 ml (2% Lidocaine, 1 :80000 epinephrine); Septodont, Saint –Maur-des-Fosses, France
E	Composite Material	Filtek Supreme XTE, 3M Espe, USA
F	Sodium Hypochlorite $Ca(OH)_2$	2.5% Sodium Hypochlorite, Milton; Procter & Gamble Australia, Parramatta, NSW
G	Non-setting Calcium Hydroxide	Ultracal; Ultradent; South Jordan
H	Intermediate restorative material	IRM; Dentsply, Milford, DE, USA
I	Gutta Percha (GP)	Densply, Maillefer, Ballaigues, Switzerland
J	Root canal sealer	Roth Sealer; Roth International Ltd; Chicago, USA

TREATMENT UNDERTAKEN

Visit 1 (22-9-2015)

- AH attended all the visits with his mother
- Full clinical and radiographic examination
- Provisional treatment plan agreed
- The maxillary incisors were fractured, with temporary restorations in situ
- The patient was not in pain

Visit 2 (2-11-15)

- AH was very nervous on first treatment visit, due to a previous painful experience at the dentist
- Vitality tests completed
- <u>UR1 endodontic treatment</u>
    - Inhalation sedation (30% Nitrous oxide and 70% Oxygen, titrated)
    - Topical and 1.8ml 2% lidocaine with adrenaline 1:80000
    - Rubber dam isolation
    - UR1 access cavity
    - Removal of necrotic pulp tissue
    - Sodium Hypochlorite irrigation
    - Working length radiograph (UR1=21mm)
    - $Ca(OH)_2$ to 21mm and GIC temporary restoration
    - NPBM and Inhalations sedation helped the patient accept the treatment
- Frankl: 2

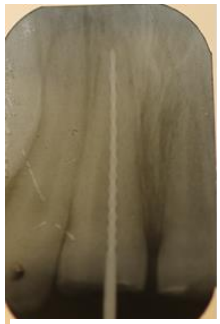

Figure 8: Working length radiograph UR1 (2-11-2015)

Visit 3 (8-12-15)

- No complaints
- Redone vitality tests – inconclusive results

Test	*UR2*	*UR1*	*UL1*	*UL2*
EPT	27	-	46	59
Ethyl Chloride	+ve	-ve	-ve	+ve
TTP	-	-	-	-
Colour	-	v. dark	-	-

- <u>UL1 endodontic investigation</u>
    - Inhalation sedation
    - Local anaesthetic and rubber dam placed as explained above
    - Removed GDPs restoration – into pulp chamber
    - No pulp tissue, no bleeding, no odour or exudate
    - Working Length at 21mm using apex locator
    - Sodium Hypochlorite irrigation

- $Ca(OH)_2$ dressing to 21mm and GIC liner
- Composite build-up with crown form and A1B shade

- Unable to complete polishing due to patients challenging behaviour
- Frankl: 2

Visit 4 (3-2-16)

- No signs or symptoms
- <u>Composite Build-up of UR1</u>
  - Inhalation sedation
  - Local anaesthetic and rubber dam used
  - Composite build-up with crown form and A1B shade
  - Polished
- Frankl: 3

Visit 5 (25-2-16)

- UL1 mild TTP, no other complaints
- <u>$Ca(OH)_2$ change UR1, UL1</u>
  - Inhalation sedation and local anaesthetic
  - Rubber dam used
  - Access cavities opened on both UL1, UR1
  - Working length after composite build ups (UR1,UL1=24.5mm). Confirmed with apex locator.
  - Sodium Hypochlorite irrigation
  - Dried and $Ca(OH)_2$ dressing to 24.5mm
- Frankl: 3

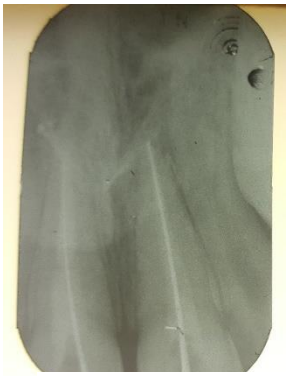

Figure 9: Working length radiograph UR1,UL1 (25-2-2016)

Visit 6 (22-3-2016)

- UL1 is still tender, decision not to obturate today
- <u>UR1 obturation</u>
  - Inhalation sedation and local anaesthetic
  - Access cavity opened on UR1
  - Canal irrigated, dried, apical stop present
  - UR1 obturated with cold lateral condensation GP and root canal sealer
  - Post operative radiograph shows good obturation
- Frankl: 3

Visit 7 (28-6-2016)

- No complaints, UL1 symptoms have settled, UR1 no SOS
- <u>UL1 obturation</u>
  - Inhalation sedation and local anaesthetic
  - Access cavity opened on UR1
  - Canal irrigated, dried, apical stop present
  - UL1 obturated with cold lateral condensation GP and root canal sealer
  - Post op radiograph shows good obturation
- Plan for whitening both UR1/UL1
- Impression for whitening tray done
- Frankl: 4

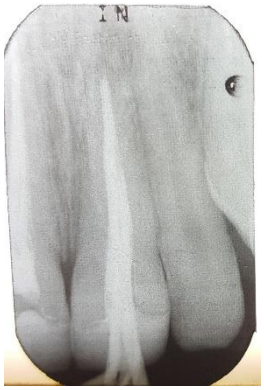

Figure 10: Obturation of UL1 (28-6-2016)

Visit 8 (18-10-16)

- Rubber dam isolation
- UR1,UL1 prepared for inside outside bleaching
- Reduced GP to below CEJ, GIC lining placed
- Preoperative photographs and shade taken
- Carbamide peroxide 10% provided with instructions
- Four month review radiograph taken
- Frankl: 4

Visit 9 (25-10-16)

- Both patient and mother happy with bleaching result
- Access cavities restored with composite under rubber dam

POST-TREATMENT PHOTOGRAPHS

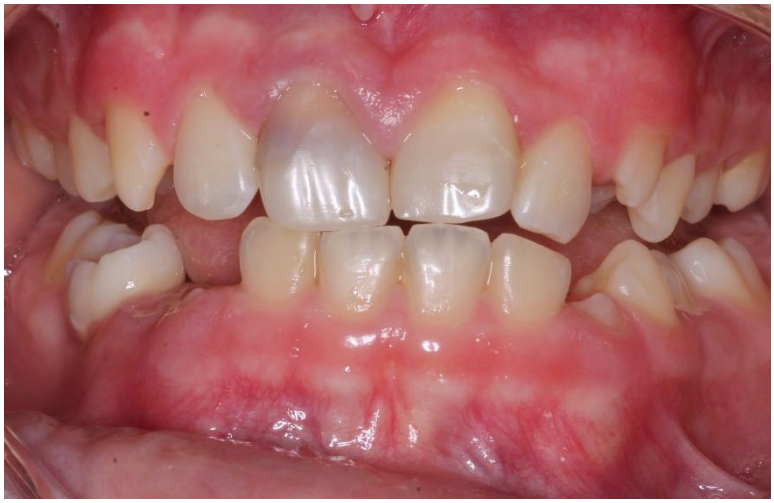

Figure 11: Pre whitening 18-10-2016

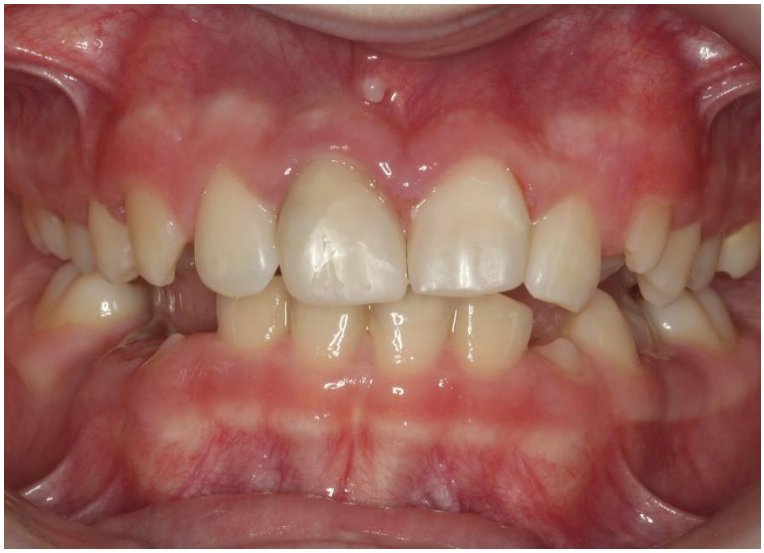

Figure 12: Post whitening 25-10-2016

POST-TREATMENT RADIOGRAPHS

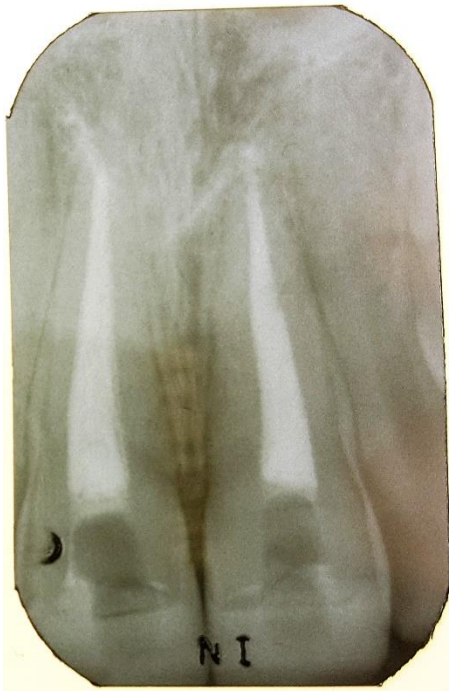

Figure 13: Four month review radiograph (18-10-2016)

Radiographic findings

- Well condensed root canal treatment on maxillary central incisors
- Some calcium hydroxide extruded on UR1
- No signs of root resorption

LONG TERM TREATMENT PLAN AND FUTURE CONSIDERATIONS

- Clinical and radiographic review
- Prevention
- Orthodontic assessment

DISCUSSION AND REFLECTION ABOUT CASE PRESENTED

AH sustained an enamel dentine pulp (EDP) fracture on both the maxillary central incisors.

These injuries are common and studies quote an estimated 5.3-21% of children will have dental trauma, with the most affected group being 7-12 year old[1,2].

Behaviour management

AH's previous bad experience made behaviour management challenging. The case demonstrates how the use of NPBMT together with inhalation sedation helped in completing the treatment and provided a positive dental experience[3]. In the final appointments, AH was able to accept treatment without inhalation sedation.

Enamel dentine pulp fracture

The principle of early restoration on EDP fractures is to preserve pulp vitality. In this case the maxillary central incisors were non vital on presentation hence endodontic treatment was the only option.

Implications of possible additional periodontal injury

Although we are not certain what the periodontal injury was due to the late presentation, it can be assumed that either a concussion or a subluxation occurred as the teeth had not moved position. These two periodontal injuries are the most common. If this was an injury involving only the periodontium the prognosis would be very good with over 80% of teeth remaining vital[4]. However, as the injury also involved an EDP fracture vitality was lost on both maxillary central incisors. The absence of any root resorption indicates that the trauma to the periodontium was minimal. Therefore it appears that the loss of vitality was primarily due to the crown fracture.

Treatment

The patient presented five months after the initial injury. The teeth were non vital. On the first visit baseline radiographs and vitality tests were done[5]. The treatment plan was to build up the maxillary incisors with composite to improve aesthetics and provide an effective seal for the endodontic treatment. The aim of early restoration is also to provide a consistent reference point for diagnosis of the working length. This was achieved radiographically and using an apex locator. The UR1 and UL1 were extirpated and built up over two separate appointments as the patient had limited cooperation. As both teeth do not appear fully developed apically, apexification was

achieved using calcium hydroxide before obturation. Prolonged use of $Ca(OH)_2$ as intracanal dressing has been shown to weaken the tooth structure and in such cases an apical barrier can be formed with the use of MTA. In our case the apices were almost closed and for that reason, after three months of $Ca(OH)_2$ dressing, the UR1 was ready for obturation with cold lateral condensation. Due to recurrent infection the UL1 obturation was delayed for a further 3 months.

Overall the case has been successful, we have build up the confidence of AH, as well as restored the maxillary central incisors.

LESSON LEARNED

- Successful outcomes following dental trauma depend on prompt and appropriate management
- Management of dental trauma maybe the patients first experience of dentistry. Use of NPBM and inhalation sedation can aid successful outcomes.

REFERENCES

1. Güngör HC. Management of crown-related fractures in children: an update review. *Dent Traumatol*. 2014;30(2):88-99.
2. Glendor U. Epidemiology of traumatic dental injuries - A 12 year review of the literature. *Dent Traumatol*. 2008;24(6):603-611.
3. Hosey MT. UK National Clinical Guidelines in Paediatric Dentistry. Managing anxious children: the use of conscious sedation in paediatric dentistry. *Int J Paediatr Dent*. 2002;12(5):359-372.
4. Andreasen. dentaltraumaguide. www.dentaltraumaguide.org..
5. DiAngelis AJ, Andreasen JO, Ebeleseder KA, et al. International Association of Dental Traumatology guidelines for the management of traumatic dental injuries: 1. Fractures and luxations of permanent teeth. *Dent Traumatol*. 2012;28(1):2-12.
6. Andreasen JO, Munksgaard EC, Bakland LK. Comparison of fracture resistance in root canals of immature sheep teeth after filling with calcium hydroxide or MTA. *Dent Traumatol*. 2006;22(3):154-156.

Case Number 3

Complex Case 1

Nikolaos Lygidakis

Eastman Dental Institute
University College London
2017

CASE SUMMARY

UO is a 10-year-old fit and well girl who was referred by her community dentist for management of enamel defects.

Clinically there was generalized hypoplasia affecting all the teeth and no post eruptive breakdown was seen. There also was caries affecting multiple teeth.

A diagnosis of Hypoplastic Amelogenesis Imperfecta and dental caries was made based on the clinical and radiographic findings.

Treatment included preformed metal crowns (PMCs) on all the first permanent molars, composite restoration of multiple teeth and extraction of UL7. Restorations were carried out using non-pharmacological behaviour management techniques.

PATIENT DETAILS

Initials: UO
Gender: Female
Age at start of treatment: 10 years
Age at last review: 12 years

PRE-TREATMENT ASSESSMENT

HISTORY OF PRESENTING COMPLAINT

UO was referred by the community dentist for the management of the affected Permanent teeth. UO had no pain or sensitivity on presentation, but was concerned about the appearance.

RELEVANT MEDICAL HISTORY

Fit and well, born at 38 weeks, no known allergies, no medical conditions and up to date vaccinations.

DENTAL HISTORY

UO was a regular attender at the community dental service for checkups and treatment. She previously had restorations under local anaesthetic.

FAMILY HISTORY

Lives with her mother, father and three sisters. Somalian origin. No other family members with dental anomalies.

SOCIAL HISTORY

Year 6 at school.

DIET

Good eater, has fruit and vegetables regularly.

ORAL HYGIENE

Brushes twice daily with adult toothpaste and an electric toothbrush.

HABITS

No habits.

CLINICAL EXAMINATION

EXTRA-ORAL EXAMINATION

- No facial asymmetry
- No lymphadenopathy
- No TMJ abnormalities

INTRA-ORAL EXAMINATION

- Soft tissues – healthy appearance
- Oral hygiene – fair with some plaque deposits present
    - Simplified plaque index – 1.00 (OHI-S) (Greene and Vermillion 1964)
    - BPE

1	1	1
1	1	1

- Hard tissues
    - Teeth present

7	6	5	4	3	2	1	1	2	3	4	5	6	7
7	6	5	4	3	2	1	1	2	3	4	5	6	7

    - Carious teeth

		6			4						4	5	6	7
		6											6	7

    - Existing amalgam restorations on first molars and composite restoration on maxillary incisors
- Occlusion
    - Class I occlusion
    - Posterior crossbite UR5,UR6
    - Mandibular midline shift to the right
    - Well aligned arches with no crowding

- Amelogenesis imperfecta
    - The hypoplastic phenotype affecting all the teeth in the permanent dentition has a characteristic concave appearance on the mandibular incisors and a generalized rough and pitted appearance on all the teeth. An element of hypomineralisation may also be present due to the white chalky enamel present which lacks translucency.

PRE-TREATMENT PHOTOGRAPHS

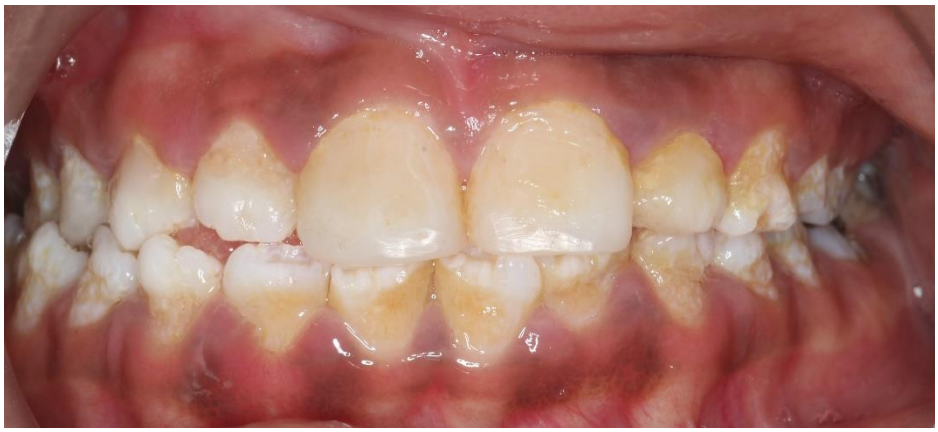

Figure 14: Anterior view 19-4-2016

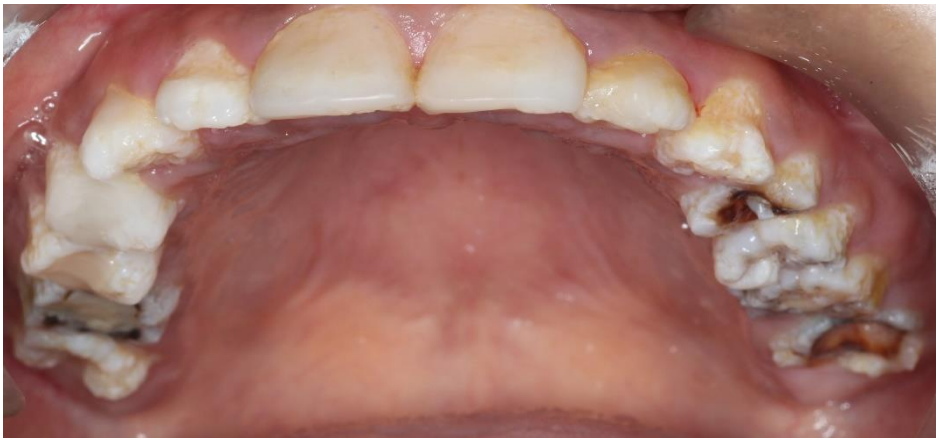

Figure 2: Maxillary occlusal view 19-4-2016

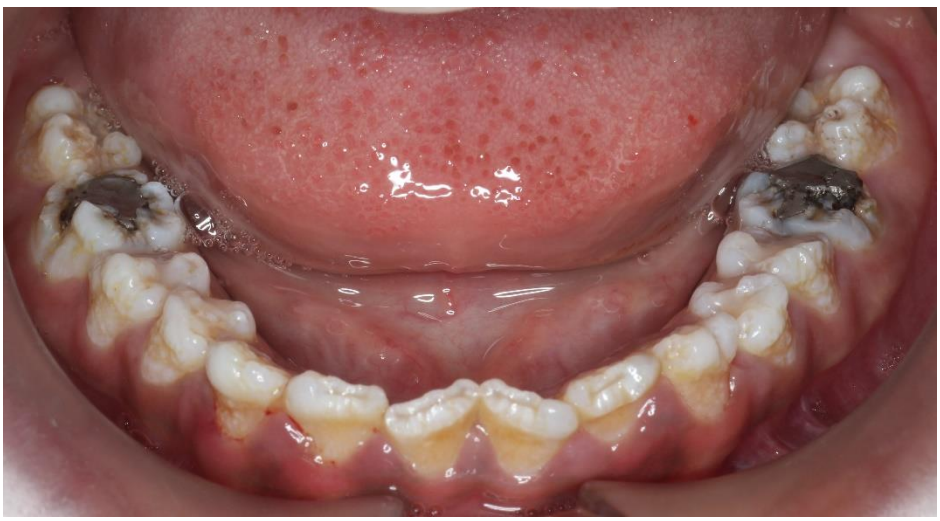

Figure 3: Mandibular occlusal view 19-4-2016

GENERAL RADIOGRAPHIC EXAMINATION

Radiographs taken:

- Bitewings
- Orthopantomogram

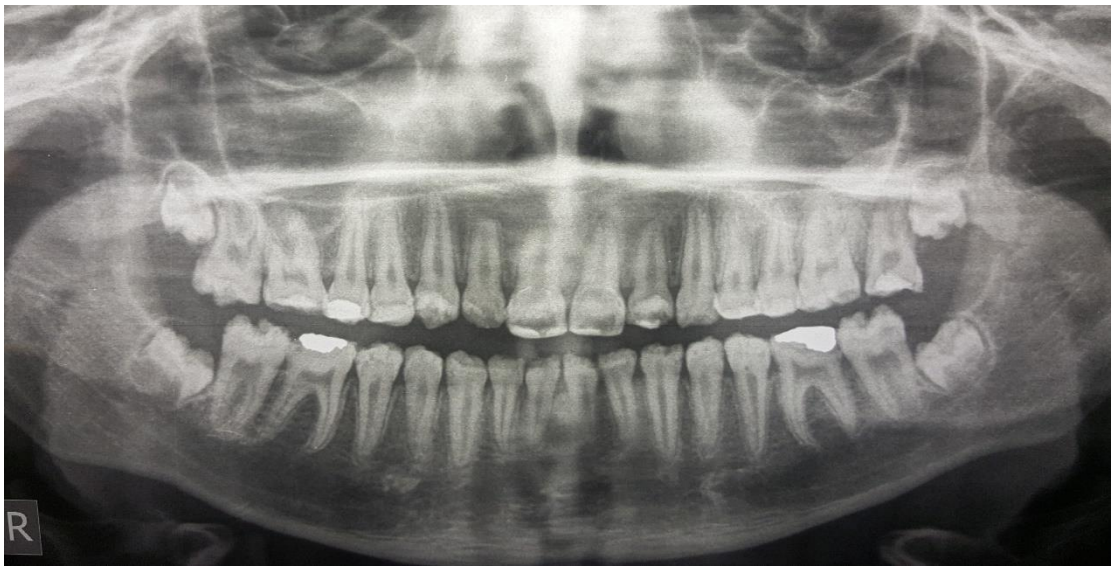

Figure 4: Orthopantomogram 19-4-2016

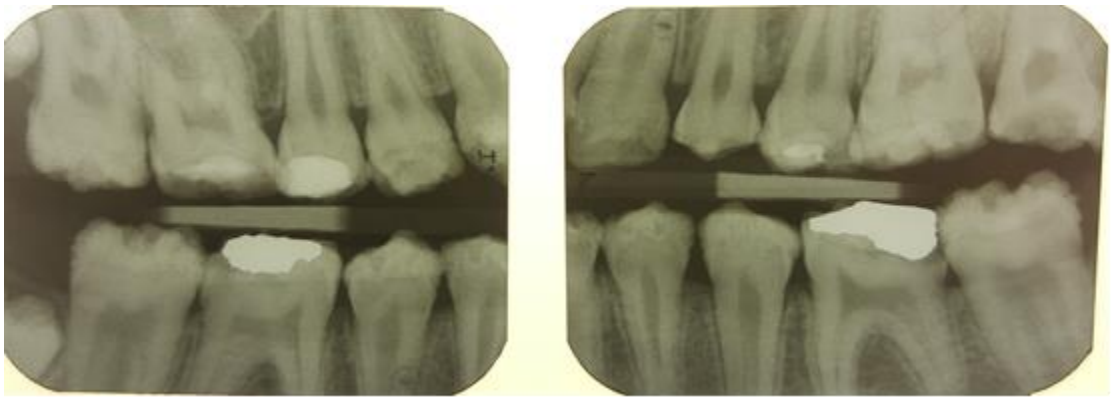

Figure 5: Horizontal Bitewing Radiographs 18-6-2015

Radiographic findings:

- OPG
  - All permanent teeth present
  - Typical appearance of Amelogenesis Imperfecta
- Bitewings
  - Secondary caries under amalgam restorations on mandibular first molars
  - Occlusal caries UL7, UL6, LL7

DIAGNOSTIC SUMMARY

- Hypoplastic Amelogenesis Imperfecta
- Dental caries

AIMS AND OBJECTIVES OF TREATMENT

- Restore dental caries
- Maintain dentition
- Improve aesthetics
- Monitor development of permanent dentition

TREATMENT PLAN

- Prevention
    - As outlined in the Department of Health Evidence based toolkit
- Behaviour management
    - Acclimatise and use NPBM to complete treatment
- Restorations
    - Composite build-up of the premolars and second molars
    - PMCs on the first molars
    - Extraction of the UL7
    - Re-restore maxillary anteriors
- Follow up and maintenance
    - Clinical review every 6 months
    - Reinforce OHI
    - Monitor restorations

TABLE OF MATERIALS USED

	Material used	Description
A	Topical Anaesthesia	Topical Anaesthetic Gel/Liquid – 20% Benzocaine, Dentsply, Milford, DE, USA
B	Local Anaesthesia (LA)	Lignocaine Special, 2.2 ml (2% Lidocaine, 1 :80000 epinephrine); Septodont, Saint –Maur-des-Fosses, France
C	Composite Material	Filtek Supreme XTE, 3M Espe, USA
D	Fissure sealant	Fissure Sealant Delton; Dentsply, Australia
E	Acid etch	Phosphoric Acid Gel Etchant 37.5%; Kerr Corporation, USA
F	Bonding agent	Opti bond Solo plus, single component, Total etch bonding agent, Kerr Co., California, USA
G	Preformed metal crowns (PMCs)	Stainless steel crowns; 3MTM ESPETM, St Paul, Minn, USA
H	Luting cement	Aquacem®; Dentsply, Milford, DE, USA
I	Duraphat®	Colgate Duraphat Varnish 50mg/ml dental suspension; Colgate Oral Pharmaceuticals, New York City, USA
j	Resin Modified Glass Ionomer Cement (GIC)	GC Fuji II LC; GC corporation, USA

TREATMENT UNDERTAKEN

Visit 1 (18-6-15)

- UO was referred by the community dental service for treatment of her hypoplastic teeth.
- She attended with her father for all the appointments
- Clinical and radiographic examination
    - Permanent dentition
    - Amelogenesis Imperfecta hypoplastic phenotype
    - Dental caries as charted

Visit 2 (20-10-15)

- Bitewing radiographs were taken to assess for caries.
- LL4, LL5, LR4, LR5
    - Cotton roll isolation, cleaned with pumice
    - For all composite restorations these steps were followed:
        - Brushed with pumice, etch, bond and A1B Filtek supreme XTE composite used
    - Restored the occlusal surfaces of all lower premolars
    - Occlusion was checked
- Behaviour was good
- OH poor with plaque index - 1.00 (OHI-S)
- Oral hygiene instruction (OHI) given according to the DoH toolkit (Public Health England 2014)
- Prescribed Duraphat® 2800ppm toothpaste
- Duraphat® fluoride varnish applied to all teeth

Visit 3 (17-2-16)

- No complaints
- UR4
    - Topical anaesthetic and local anaesthetic 2% lidocaine with adrenaline 1/80000
    - Rubber dam placed
    - Occlusal caries removed UR4, Composite A1 used to restore
    - Polished and occlusion checked
- Behaviour was good

Visit 4 (19-4-16)

- No complaints
- UL4 ,UL5, UL7
    - Topical anaesthetic and local anaesthetic as above
    - Rubber dam placed
    - Occlusal caries removed UL4,5,7
    - A1 composite used to restore UL4,5
    - Polished and occlusion checked
    - RMGIC placed occlusally on UL7
- OPG taken to assess condition of first molars before crowning
- Clinical photographs done
- Agreed that UL7 was unrestorable and LL6 seems to have a very deep restoration but will restore with PMC.

Visit 5 (21-4-16)

- Emergency appointment
    - Extraoral swelling left cheek extending infraorbitally, but not close to eye
    - Erythema around the diffuse swelling
    - Tenderness extraorally
    - Trismus
    - Temperature 38.5^{O}C
- After clinical and radiographic examination a diagnosis of dental abscess UL7 was made
    - Prescribed antibiotics Amoxicillin 500mg and Metronidazole 200mg orally. We were unable to do extraction due to trismus.
- Instructions given to attend A&E if swelling increases
- Review the next day

Visit 6 (22-4-16)

- Feels better than previous visit
- Has eaten
- Swelling remained similar after 3 doses of antibiotics
- To continue antibiotics and return in one week for extraction of UL7

Visit 7 (27-4-16)

- Swelling and pain resolved two days ago
- XLA UL7
    - Topical anaesthetic and local anaesthetic as above
    - UL7 Elevated and extracted intact
    - Post-operative instructions given and Haemostasis achieved
- Patient did very well – praised

Visit 8 (24-5-16)

- Attends with father
- No complaints, however amalgam filling LL6 has come out
- PMC on UL6
    - Topical anaesthetic and local anaesthetic as above
    - LL6 caries removed with excavator
    - Size 4 PMC cemented on UL6 using aquacem
    - LL6 temporized with RMGIC FUJI II
- Duraphat® fluoride varnish applied to all teeth

Visit 9 (8-6-2016)

- Attends with father
- No complaints
- PMC on LR6 and composite on LR7
    - Topical anaesthetic and local anaesthetic as above
    - Rubber dam isolation
    - Caries removed from LR7
    - Composite occlusal restoration A1B LR7
    - Amalgam restoration and occlusal caries removed from LR6, GIC lining placed
    - Size 4 PMC cemented on LR6 using Aquacem

Visit 10 (11-10-2016)

- No complaints
- PMC LL6 and composite on LL7
    - Topical anaesthetic and local anaesthetic as above
    - Rubber dam isolation
    - All caries removed from LL7
    - Composite occlusal restoration A1B LL7
    - Polished
    - Distal preparation LL6 for PMC, GIC lining placed and size 2 PMC cemented with Aquacem

Visit 11 (18-10-2016)

- No complaints
- PMC UR6 and fissure sealant on UR7
    - Topical anaesthetic and local anaesthetic as above
    - All caries removed from UR6
    - Fissure sealant placed on UR7
    - Size 3 PMC cemented on UR6 with Aquacem
- Prescribed Duraphat 2800ppm toothpaste
- Duraphat® fluoride varnish applied to all teeth
- Oral hygiene improved with plaque index at 0.66 (OHI-S)
- Review in 3 months

Visit 12 (17-12-2016)

- No complaints
- UR1, UL1
    - Topical anaesthetic and local anaesthetic as above
    - Rubber dam placed
    - Old restorations removed from UL1, UR1, Composite A1B used to restore with crown form
    - Polished and occlusion checked

Visit 13 (23-01-2017)

- No complaints
- UR2, UL2
    - Topical anaesthetic and local anaesthetic as above
    - Rubber dam placed
    - Old restorations removed from UL2, UR2, Composite A1B used to restore with crown form
    - Polished and occlusion checked

Visit 14 (01-02-2017)

- No complaints
- UR3, UL3
    - Topical anaesthetic and local anaesthetic as above
    - Rubber dam placed
    - Old restorations removed from UL3, UR3, Composite A1B used to restore with crown form
    - Polished and occlusion checked
- Very happy with result
- Post-operative photographs done

POST-TREATMENT PHOTOGRAPHS

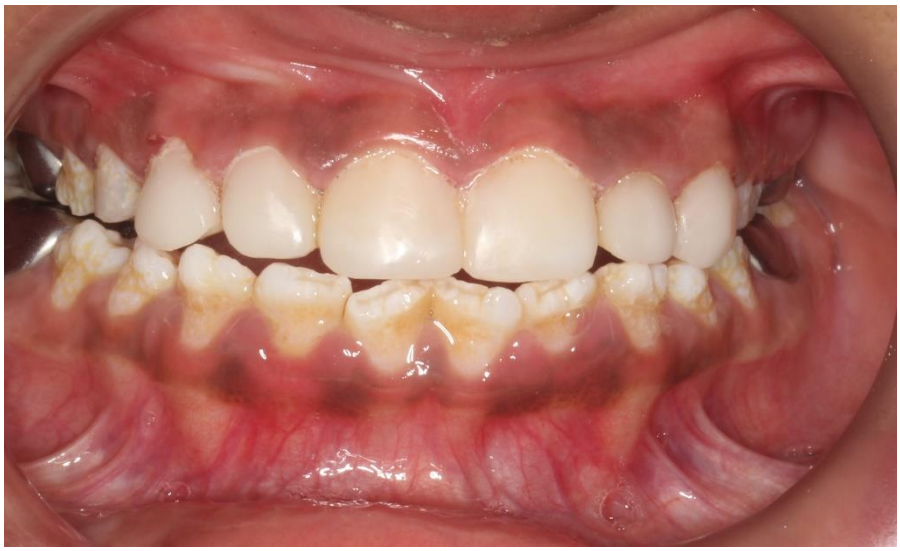

Figure 6: Anterior view 1-2-2017

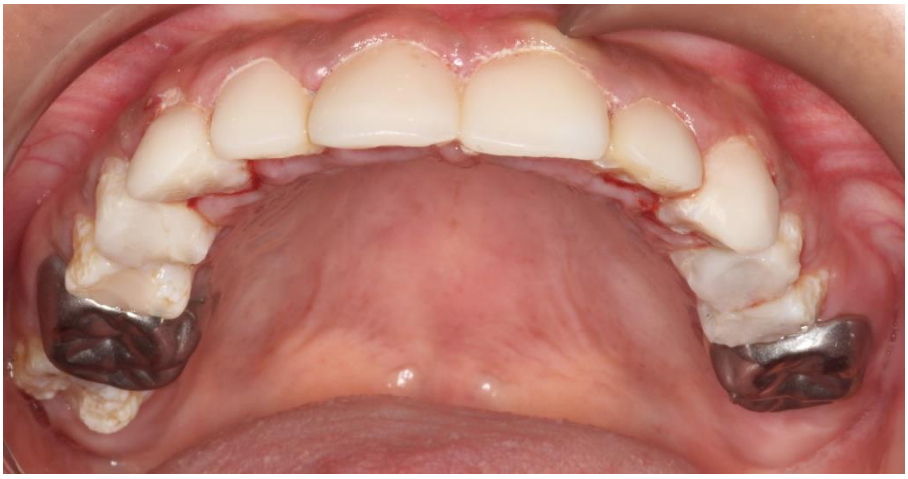

Figure 7: Maxillary occlusal view 1-2-2017

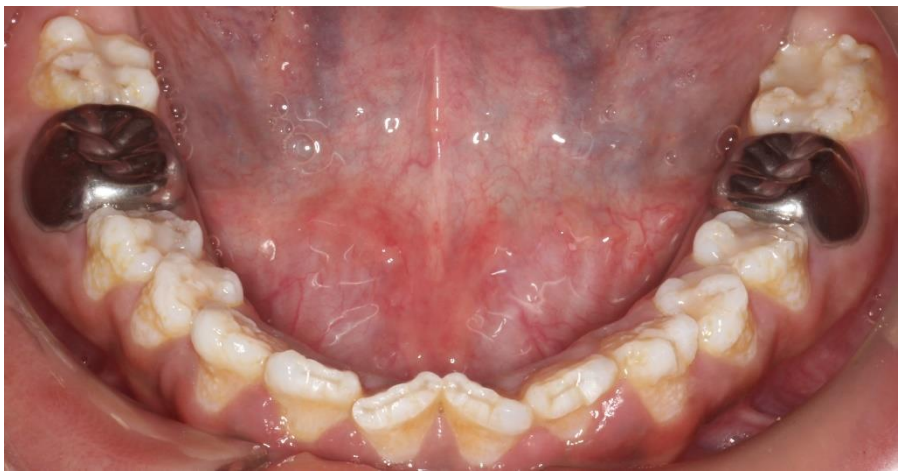

Figure 8: Mandibular occlusal view 1-2-2017

LONG TERM TREATMENT PLAN AND FUTURE CONSIDERATIONS

- Review every 6 months
- Prevention
- Fluoride varnish every three months

DISCUSSION AND REFLECTION ABOUT CASE PRESENTED

Amelogenesis imperfecta (AI) represents a group of conditions which affect the structure and appearance of enamel. It affects 1:700 to 1:14000 people (Crawford et al. 2007).

The classification of Winter and Brook in 1975 is one of the most accepted ones (Crawford et al. 2007). UO had a hypoplastic type of AI which presented with random pits and was also rough. UO seems to be the first in her family with a dental anomaly, so we were unable to do a pedigree chart.

When considering the diagnosis it is important to consider the differentials. Chronological hypoplasia is the main differential diagnosis in our case, however this can be excluded as all the teeth have erupted. As part of the diagnosis the radiographic examination may be of value. In our case the OPG radiograph showed the typical appearance of AI, with taurodontism on the lower second molars.

The reasons for restoring the teeth are to improve aesthetics on the anteriors, to maintain posterior teeth and to improve the anatomy for more effective cleaning. When development has ceased heavily restored first molars can be restored with full coverage restorations (Pousette Lundgren et al. 2015).

It is important to make a correct diagnosis of hypomineralized or hypoplastic AI. As hypoplastic enamel is fully mineralized it does not break down and often no restorations are required. Also bonding is equally effective to a non AI tooth. On the other hand on a hypomineralized tooth, enamel is more prone to breakdown. UO had a hypoplastic type of AI and there was no sensitivity. However due to multiple early interventions and failure of prevention UO had a lot of caries in the posterior teeth. This is partly because of the abnormal shape of teeth as well as poor oral hygiene and diet. The anatomy of the teeth makes them more prone to caries and restorations may allow improved cleaning. Additionally the restorations on the anterior teeth were breaking down and were unaesthetic. Although the teeth were predominantly hypoplastic an element of hypomineralisation was also possibly present due to the chalky appearance enamel which chipped off easily.

UO's oral hygiene improved overall but was still not perfect. A preventive regime was formulated according to the Deparment of Health Toolkit (Public Health England 2014).

Overall we have succeeded in managing UO well and maintaining her dentition apart from the loss of the UL7 due to unrestorable caries. She has also been educated towards better oral hygiene habits.

LESSONS LEARNED

- Amelogenesis imperfecta is a complex condition to treat
- Early diagnosis and appropriate planning is essential

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Case Number 4

Complex Case 2

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2017

CASE SUMMARY

HHL was an 8 years old boy who was referred by the orthodontist to the Eastman Dental Hospital for management of his malocclusion and missing teeth.

The diagnosis of hypodontia was made as well infraoccluded primary teeth on a class III malocclusion.

HHL was medically fit and well. He did not have any complaints regarding his teeth. HHL had a class III malocclusion on a mild Class III skeletal base. Intraorally he was in the mixed dentition with fair oral hygiene. The lower arch had mild crowding and the lower right first primary molar and lower second primary molars were severely infraoccluded. The upper arch also had mild crowding.

The OPG demonstrated the congenital absence of all four second premolars and the lower right first premolar. In addition, it showed resorption of the distal roots of the upper second deciduous molars by the upper first permanent molars.

After the orthodontic/paediatric meeting it was decided that HHL would benefit from interceptive orthodontics. This would involve correction of the anterior local crossbite and extraction of the retained upper second deciduous molars to allow for the eruption of permanent molars. In addition, the infraoccluded lower deciduous molars would be build up with composite into the occlusion.

Treatment was carried mainly using non–pharmacological behaviour management (NPBM) techniques. Treatment provided:

- Prevention and acclimatization
- Oral hygiene instructions (OHIs).
- Dietary advice.
- Upper removable appliance to correct the crossbite by the orthodontic department
- Restorations:
    - Composite build ups of the lower right primary molars and the lower left second primary molar

PATIENT DETAILS

Name: HHL

DOB: 28/10/06

Age: 9 years on presentation

Sex: Male

First attended: 13/10/2015

REASON FOR ATTENDANCE

Referred by orthodontist to manage the infraoccluded primary teeth.

CHIEF COMPLAINT

HHL is aware that he was missing teeth but is not concerned about it.

MEDICAL HISTORY

Fit and well, no know allergies and not on any medication. There is a history of tonsillectomy and adenoidectomy.

DENTAL HISTORY

Regular attender to GDP every 6 months. Has never had local anaesthetic or any restorations.

FAMILY AND SOCIAL HISTORY

HHL is adopted and has no siblings. He attends school and is currently in year 4. No information was known about hypodontia of the parents.

DIET

Good eater, drinks water mainly and as snacks usually has cheese and toast.

ORAL HYGIENE

Good oral hygiene, brushes twice daily with fluoridated adult toothpaste and manual toothbrush.

HABITS

No habits.

CLINICAL EXAMINATION

EXTRA-ORAL

- No facial asymmetry
- No lymphadenopathy
- No TMJ abnormalities

INTRA-ORAL

- Soft tissues – health appearance
- Oral hygiene – good with no minimal plaque deposits present.
    - Simplified plaque index – 12% (Greene and Vermillion 1964)
- Hard tissues
    - Mixed dentition
    - LRE,D, LLE severely infraoccluded
    - Teeth present

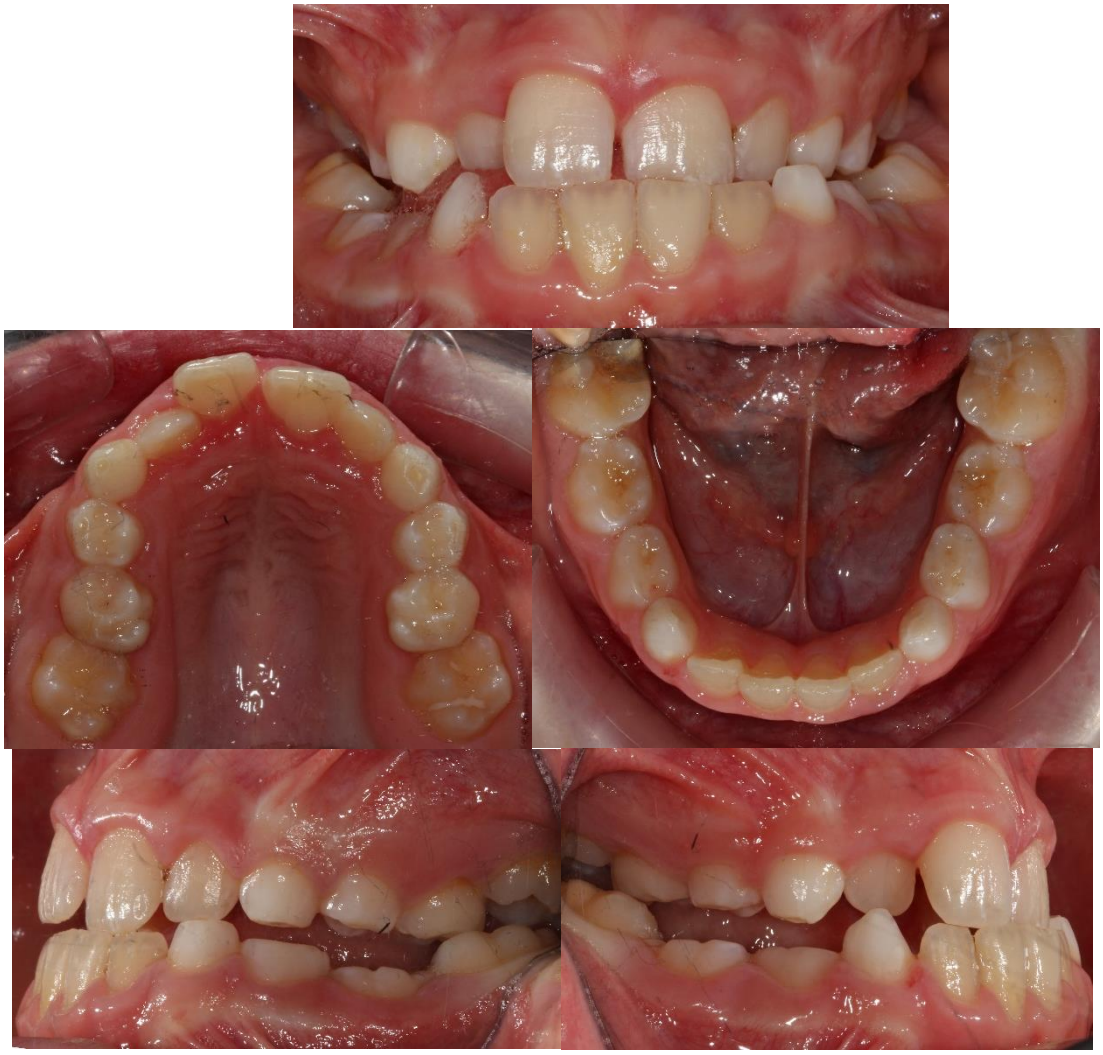
6	E	D	C	2	1	1	2	C	D	E	6
6	E	D	C	2	1	1	2	C	D	E	6

- Occlusion
    - Class III malocclusion with increased vertical proportions
    - Posterior bilateral crossbite and anterior localized crossbite of UL1

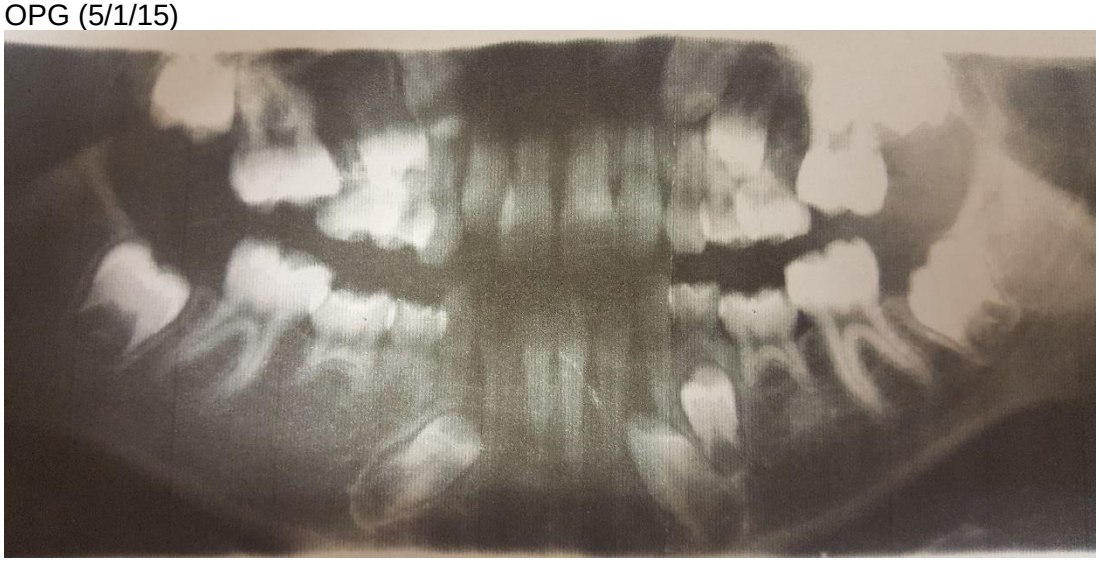
Radiographically there are five permanent teeth missing, including all the second premolars and the lower right first premolar.

PREOPERATIVE IMAGING

Intraoral photographs (9/6/15)



OPG (5/1/15)



- Absence of all four second premolars and the lower right first premolar
- Resorption in the distal roots of the upper second deciduous molars
- Third molars not visible yet

DIAGNOSIS AND TREATMENT PLANNING

DIAGNOSIS

1. Dentition
    - Hypodontia, infraocclusion of LRD,E LLE
- Orthodontic
    - Class III malocclusion on class III skeletal base
    - Upper and lower arch mild crowding
    - Posterior and anterior crossbite

TREATMENT OBJECTIVES

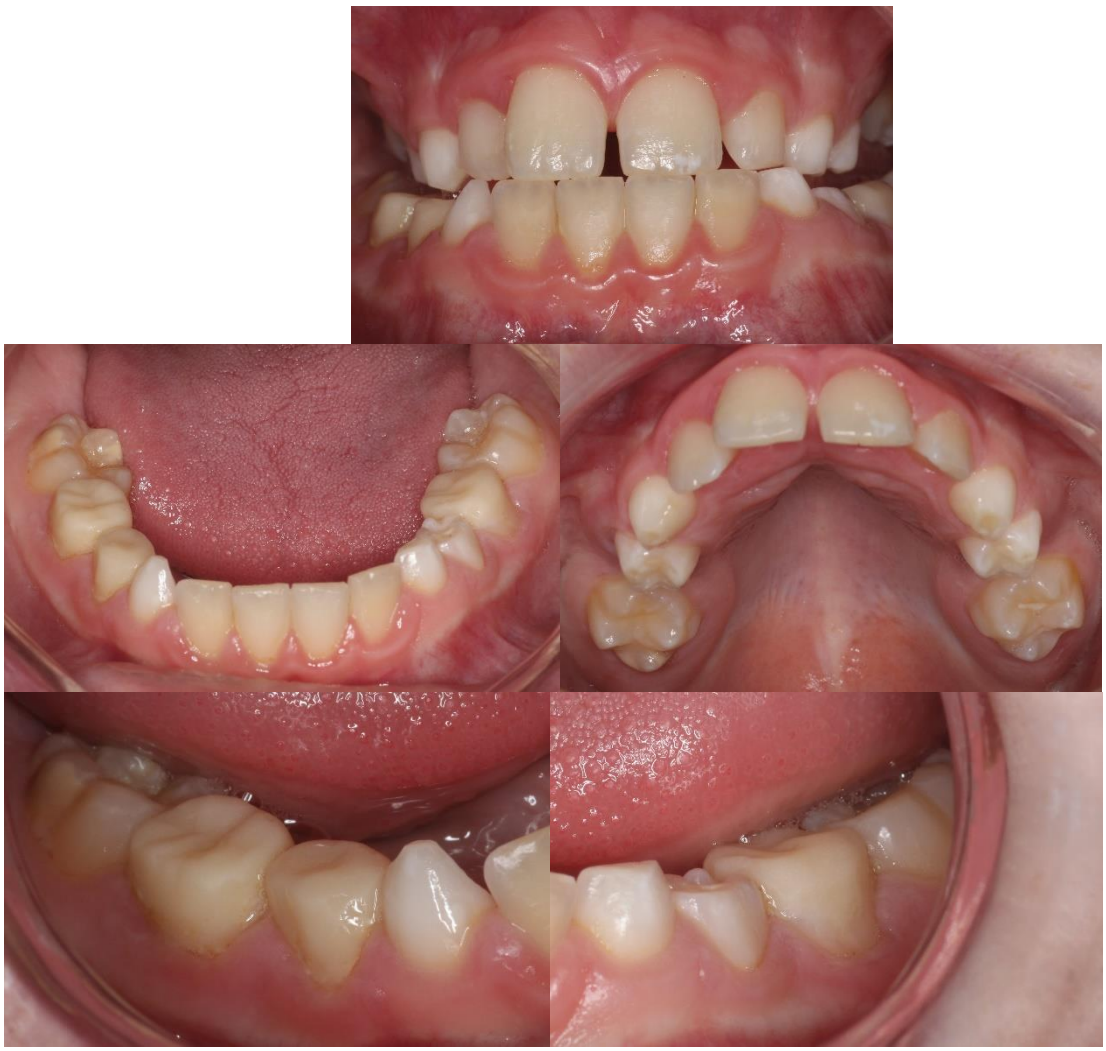
- Align dentition
- Build up and maintain infraoccluded primary teeth
- Correct crossbites

PROVISIONAL TREATMENT PLAN

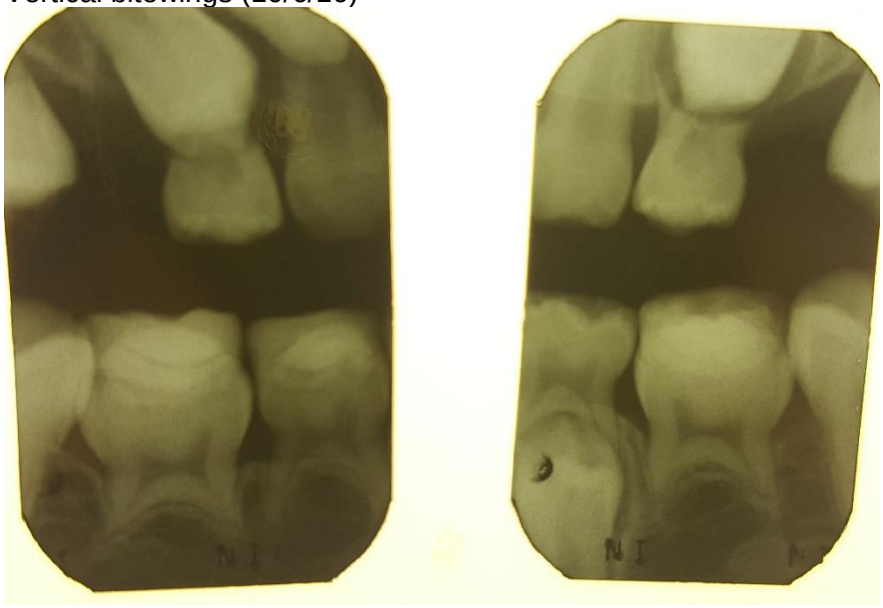
1. Prevention
    - OHI
    - Fluoride varnish application twice yearly
2. Behaviour management
    - Acclimatise and use NPBM to complete treatment
3. Restorations
    - Composite build-up of the lower right primary molars and the lower left second primary molar
4. Orthodontic
    - Orthodontic department to provide upper removable appliance to correct crossbite
5. Follow up and maintenance
    - Clinical review every 6 months
    - Reinforce OHI
    - Monitor composite build ups

POSTOPERATIVE IMAGING

Intraoral photographs (10/6/16)



Vertical bitewings (10/6/16)



- Composite buildups to occlusal plane LRE, LRD, LLE
- Root resorption present on LLE, LRE, LRD
- ULD, URD close to exfoliation
- Absent LR5,LR4,LL5
- Grade 1

TREATMENT PROGRESS AND DENTAL MANAGEMENT

9-3-15 Visit 1

HHL was referred from his local orthodontist to our orthodontics department. He attended with his foster mother. He was assessed and a treatment plan was formulated. This was to:

- GDP to extract the upper retained Es
- Paediatric dental department to build up infraoccluded lower Es and Ds
- URA to correct crossbite
- Reassessment

13-10-15 Visit 2 (first visit to paediatric deparment)

- HHL attended with his foster mother.
- He was fit and well with no known drug allergies and no medical conditions.
- A new patient assessment was done on the day and a below are the findings:
    - UL1 in crossbite
    - Class III malocclusion
    - LRD,E, LLE severely infraoccluded
    - Small area of hypomineralisation on LR6
    - Small white spot UL1 incisally
    - OH good with PI at 12%
- The initial referral plan by orthodontics was to build up all lower Es and Ds but on examination the LLD was not infraoccluded and a permanent successor was present. Hence we agreed to build up the lower Es and the LRD.
- Options were discussed for the treatment and it was decided to attempt a technique were the build up is done with chemical cure composite under a preformed metal crown and then the PMC is removed.

9-3-16 Visit 3

HHL attended with his foster father. There were no complaints.
The general dental practitioner had extracted the Upper Es.
He was wearing a URA provided by our orthodontic deparment.
Plan to do build up the LRD with composite

- LRD composite build up
- Cotton roll isolation
- Size 2 crown chosen

- Core build up of LRD with A1 Filtek composite
- Cemented PMC with chemical cure composite
- Waiting time of four minutes
- Crown sectioned mesiocclusally
- Polished composite
- Separators placed interproximally for crowns on the Lower Es at the next visit
- Good cooperation
- OHI and TBI done

23-3-16 Visit 4

HHL attended with his foster father. There were no complaints and the crown on the LRD was intact.

Plan to do build up with composite the lower Es

- Separators removed
- Cotton roll isolation
- Size 2 crown chosen
- Core build up of lower Es with A1 Filtek composite
- Cemented PMC with chemical cure composite
- Waiting time of four minutes
- Crown sectioned mesiocclusally
- Final finishing done and occlusion checked. Patient and father very happy with final appearance.
- Good cooperation

Following the completed treatment, the lower Es and LRD were on the same occlusal level as the remaining teeth and in occlusion with the upper teeth.

10-6-16 Visit 5

HHL attended with his foster father. There were no complaints and all the crowns were intact. He was still continuing with the URA.

- Clinical examination showed the intact crowns present
- Oral hygiene was good with PI at 12%
- Clinical photographs done
- Post op vertical bitewing radiographs done

Plan to review in six months, then discharge back to orthodontics for his ongoing care.

APPRAISAL AND DISCUSSION

Infraocclusion is a frequent clinical finding having a prevalence of 1.3% to 8.9% in primary molars. In 17% to 25% of the cases, they are associated with missing permanent premolars (Kennedy, 2012; McGeown & O'Connell). Clinicians treating patients with infraocclusion need to make a decision whether to follow an extraction or retention plan.

In the majority of cases where infraocclusion is not associated with hypodontia the primary molar will be left to exfoliate or extracted. There are however cases where the severity of infraocclusion, the position and resorption rate of the root, the number of missing teeth and the overall orthodontic evaluation will lead to a treatment plan which requires retention of the primary teeth.

Hypodontia is the term used for the congenital absence of teeth. It is common affecting 3.5-6.5% of the population in the permanent dentition and 0.1-0.9% in the primary dentition(Brook, 1974; Thilander & Myrberg, 1973). It has been described as hypodontia when 1-5 teeth are missing, oligodontia when 6 or more teeth are missing and anodontia when all teeth are missing. The third molars are not included in the above. Furthermore, hypodontia can be as part of a syndrome or non-syndromic. The main syndrome associated with hypodontia is Ectodermal Dysplasia, but also Oro-Facial Digital syndrome, Down's syndrome and syndromes associated with oral cleft. Non syndromic is usually autosomal dominant inheritance and some genes such as MSX1, PAX9 have been associated with hypodontia. In our case HHL did not have any features of ED or any other syndrome. Taken into account that he is adopted the foster parents did not have any information of parental hypodontia, however we assume that this a case of non-syndromic hypodontia with five missing permanent teeth.

There are many different ways of classifying infraocclusion. It can be classified radiographically, clinically or through study models. Here we have chosen to use the simple clinical classification of Brearley and Mc Kibben from 1973 (Brearley & McKibben, n.d.). This is a commonly used system and includes the following categories:

- Slight – occlusal surface located approximately 1 mm below the expected occlusal plane for the tooth.
- Moderate – occlusal surface approximately level with the contact point of one or both adjacent tooth surfaces.

- Severe – occlusal surfaces level with or below the interproximal gingival tissue of one or both adjacent tooth surfaces.

Looking into the treatment options, in our case the 3 missing permanent lower premolars and the occlusal and skeletal profile resulted in a treatment plan requiring retention of the infraoccluded primary molars. All three of the primary lower retained molars were severely infraoccluded.

Long term studies have shown that infraoccluded primary teeth may have very slow exfoliation rates and after the age of 20 only a few teeth are lost (Bjerklin & Bennett, 2000). It is very difficult to estimate how long HHL teeth will last as there is already resorption of the roots, however once these are back in occlusion this may slow down.

Multiple different approaches have been reported in the literature for the treatment of infraoccluded primary teeth. These can be direct composite, indirect composite onlays, Preformed Metal Crowns, porcelain crowns and porcelain or gold onlays(Bonin, n.d.; Cavanaugh & Croll, 1994; Gulati & Welbury, 1998; Kennedy, 2012). All of the above have been successfully used with good results. The main advantage of the clinical approach in our case is that it enables the clinician to easily create an aesthetic crown in one appointment using materials and instruments readily available in the clinical practice and without the use of a laboratory. A previous study has shown good very good result following a 12 month review (Lygidakis, Chatzidimitriou, & Lygidakis, 2015).

Overall we were happy with how successful the treatment for HHL was. The three crown build ups were done in two visits and the whole procedure was relatively quick and easy for the patient. The end result was aesthetically very good and brought the teeth back into occlusion. The three-month review appointment showed very good result and the patient was overall happy with the appearance and function of the restored teeth.

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