INTRA-ORAL DELIVERY SYSTEM FOR

ANTIFUNGAL RELEASE

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in the Faculty of Medical and Human Sciences

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<td>Degree of Conversion</td>
</tr>
<tr>
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<td>DiMethyl SulphOxide</td>
</tr>
<tr>
<td>F</td>
<td>Force</td>
</tr>
<tr>
<td>FLUc</td>
<td>Fluconazole powder from capsules</td>
</tr>
<tr>
<td>FLUp</td>
<td>Fluconazole pure</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform InfraRed Spectroscopy</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>I₀</td>
<td>Intensity of reference beam</td>
</tr>
<tr>
<td>I</td>
<td>Intensity of sample beam</td>
</tr>
</tbody>
</table>
MIC  Minimum Inhibitory Concentration
min  Minute
mg   Milligram
ml   Millilitre
µl   Microlitre
mm   Millimetre
MPa  Megapascal
MOPS Morpholinepropanesulfonic acid
N    Newton
NMR  Nuclear Magnetic Resonance
OD   Optical Density
PAFE Post Antifungal Effect
PBS  Phosphate buffered saline
PEM  Poly (ethyl methacrylate)
PEM/THFM Poly (ethyl methacrylate) and Tetrahydrofurfuryl methacrylate
PMMA Poly (methyl methacrylate)
R    Pearson's correlation coefficient
r    Simple correlation coefficient
r_s  Spearman correlation coefficient
S    Second
SBS  Shear Bond Strength
SD   Standard Deviation
SEM  Scanning Electron Microscopy
UV   Ultraviolet
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>VIS</td>
<td>Visible</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>YNBG</td>
<td>Yeast Nitrogen Base with Glucose</td>
</tr>
<tr>
<td>ΔE</td>
<td>Change in colour</td>
</tr>
<tr>
<td>°C</td>
<td>Centigrade</td>
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<td>§</td>
<td>Section</td>
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**ABSTRACT**

**Background:** The placement of removable dental prostheses produces significant changes in the oral environment that may lead to adverse effects on the integrity of the oral tissues. Denture-induced candidosis, caused by candidal infection of the palatal mucosa, is the most frequent complication (40 %) in removable denture wearers. It predominantly affects immunosuppressed and medically compromised patients. In these high-risk patients the oral cavity may provide a source for *Candida* causing systemic infection. Oral candidosis has become a significant challenge in patients with persisting risk factors and a recurrent need for antifungal treatment. In addition, denture-induced candidosis is a mixed biofilm infection which provides multiple challenges for its management. Moreover, the persistent fungal colonisation on the fitting surfaces of denture often leads to cross infection and recurrence of mucosal lesions. These considerations highlight the clear need for new effective antifungal treatment modalities.

**Aims:** The aims of this project were to establish a polymeric delivery device based on denture base lining polymer, poly (ethyl methacrylate) and tetrahydrofurfuryl methacrylate (PEM/THFM), for sustained delivery of antifungal agents [chlorhexidine (CHX) and fluconazole (FLU)], for the use in the treatment of denture-induced candidosis and to test the serviceability of the lining under investigation.

**Methods:** A broth microdilution method was used to assess the spectrum of activity of the antifungal agents (CHX powder and FLU powder in two formulations pure and from capsules) against wide range of *Candida* species. Bioassay method and spectrophotometry were used to evaluate the efficiency of the PEM/THFM denture liner to release the impregnated antifungal agents and to quantify the released concentrations. Bioassay, time-kill studies and biofilm assays were used to verify the antifungal activity of the released antifungal agents. Shear bond test, water absorption, colorimetery and Fourier Transform Infrared Spectroscopy were used to test clinically important physical and mechanical properties for the impregnated liner.

**Results:** It was found that CHX has broad-spectrum antifungal activity also among *Candida* species highly resistant to FLU. Both CHX and FLU became readily leached from PEM/THFM polymer up to 4 weeks in microbiologically effective concentrations. CHX demonstrated superior antifungal efficacy against planktonic and biofilm lifestyle of *Candida* compared to FLU. Findings show that the impregnation with antifungal agents has affected all tested properties (shear bond strength, water absorption, degree of conversion and colour stability) but these changes are comparable to other long-term lining materials and are within acceptable ranges.

**Conclusions:** These findings indicate the feasibility of introducing an efficient treatment modality for candidal infections, especially denture-induced candidosis. A polymeric system containing CHX or FLU could assume a very promising treatment option as the drug is effective and directed to the site of pathology. Moreover, the distinct efficacy of CHX against *C. albicans* biofilms is a promising outcome to overcome the side effects of conventional antifungal agents and their reduced efficacy against biofilm formation.
DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Nesreen Salim

2012
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I graduated from The University of Jordan in 2003, gaining a BDS with a GPA of 3.7 out of 4 (Excellent). I worked as a teaching assistant at The University of Jordan between 2004 and 2007. After that I enrolled in a one year full-time MSc Fixed and Removable Prosthodontics program offered by The University of Manchester in 2007. I finished the degree with Distinction. Then I enrolled in a four-year clinical PhD (Doctor of Clinical Dental Science in Fixed and Removable Prosthodontics) in 2008. In 2010 I won the Friends of the Hebrew University Prize for the best oral presentation for research in the postgraduate presentation day at the School of Dentistry. I am a reviewer for Dental Materials. During the PhD I attended several scientific meetings:

- British Society of Prosthodontics meeting, in York in April 2009.
- British Society of Prosthodontics meeting, in Stirling in March 2010.
- British Society of Prosthodontics meeting, in Liverpool in April 2012.
- British Society for Medical Mycology, Cardiff, in April 2012.

In addition, some of the research work is to be presented in the following meeting:

- A poster presentation titled “Anti-biofilm activity of antifungal-impregnated denture material” was accepted and will be presented at the PER/IADR coming meeting in Helsinki in September 12-15/2012.
In addition, I have published the following papers during my studies:


I have also submitted other papers to a variety of scientific journals.
DEDICATION

IN THE NAME OF ALLAH
&
His Blessings

The all knowing, The most wise

I would like to dedicate this work to the soul of my father, who was the cornerstone of my life, the light of my eyes, the spring of my ambitions. My father, you are and will remain the source of my persistence and progress.

I would like to dedicate this work to my mother, the spring of love that was very supportive and encouraging during all stages of this work to excel towards the best. I would like to dedicate this work to my husband, who was the forefront to my achievements and without his incessant support, patience and valuable guidance this work cannot be tackled. There could not be a husband any thoughtful and benevolent more than you. This thesis is also dedicated to my dearly child Haneen.

This thesis is also dedicated to the stars of my life, brothers and sisters; Abdullah, Mohmmed, Ahmed, Mahmood, Ali and Njat, for their continuous support and tremendous encouragement.

I would like to dedicate this work to my dear friends Katayoon Azizi, Suad Othman Dua’ Jaber and Noura Nour who were with me step by step leading me down the path of even more valuable achievements by their constant support.

Finally I would also like to dedicate this work to all those people who suffered or are still suffering from injustice, aggression, physical or emotional hardships. Their pain is hoped to end one day.
ACKNOWLEDGEMENTS

By coming to the end of this scientific journey; first, I thank God, the most merciful and most gracious for edifying my mind by cognition and acquaintance.

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I also would like to thank Prof. Malcom Richardson for the language support and the critical comments he offered to me throughout writing my thesis.

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Finally, I am grateful to The University of Jordan for awarding me a studentship.
CHAPTER 1

REVIEW OF THE LITERATURE
Chapter 1

1.1 Introduction

The popular belief that edentulism will decline markedly in the future is based on epidemiologic studies (Steele et al., 2000; Muller et al., 2007). This decline is counteracted by demographic changes such as the increase in size and age of the older population and consequently the need and demand for complete dentures will increase over the next two decades as the number of older adults increases (Muller et al., 2007). In addition, the prevalence of tooth loss increases with age and the prosthetic need increases accordingly (Walter et al., 2001). Consequently there is a growing concern about denture-related mucosal pathologies (Budtz-Jorgensen, 1981; Jainkittivong et al., 2010) and, hence, providing efficient treatment of such conditions is significant.

Placement of a removable prosthesis in the oral cavity produces significant changes in the oral environment that may lead to adverse effects on the integrity of the oral tissues (Dorey et al., 1985). Mucosal reactions may result from mechanical irritation from dentures, plaque accumulation on dentures, or allergic reaction to denture base materials (Budtz-Jorgensen, 1981; Coelho et al., 2004; Freitas et al., 2008).

The adverse direct effects of wearing complete dentures include resorption of residual ridges and also pathological changes of the oral mucosa in the form of denture-induced candidosis, flabby ridges, angular cheilitis, denture hyperplasia, burning mouth syndrome and traumatic ulcers (Dorey et al., 1985; Jainkittivong et al., 2010). These adverse consequences often result in patient discomfort, unstable occlusion, and insufficient masticatory function (Dorey et al., 1985; Jainkittivong et al., 2010). In addition systemic or general diseases may alter the tissue response
resulting in oral lesions, so these lesions may indicate a serious underlying disease (Budtz-Jorgensen, 1981; Khatibi et al., 2011).

Denture-induced candidosis (Candida-associated denture stomatitis, denture-induced stomatitis), characterised by candidal infection of the palatal mucosa, is the most frequent complication of wearing complete or partial dentures (Budtz-Jorgensen, 1981; Jainkittivong et al., 2010). The exact prevalence of denture-induced candidosis is unclear; it has been reported in 45-70% of complete denture wearers (Figueiral et al., 2007; Dagistan et al., 2009). Moreover, denture-induced candidosis is the most common clinical presentation of oral candidosis (Samaranayake et al., 2009). This has driven research towards this research area, which has focused attention on effective means to control these lesions (Cawson, 1963; Giuliana et al., 1997; Dhir et al., 2007).

Denture-induced candidosis is described as an inflammatory process of the mucosa of denture-bearing tissues. This condition is almost invariably asymptomatic (Budtz-Jorgensen, 1974), usually affects the hard palate (Budtz-Jorgensen, 1981) and frequently is associated with angular cheilitis and glossitis (Ritchie et al., 1969; Budtz-Jorgensen and Bertram, 1970a). Denture-induced candidosis is seen more frequently among women than men (Arendorf and Walker, 1980). There are a number of factors that can give rise to denture-induced candidosis such as trauma, allergy, and dietary factors. It is a multifactorial disease with Candida albicans (C. albicans) being the primary aetiological agent (Olsen, 1974; Coco et al., 2008).

Denture-induced candidosis may heal partially or completely after topical antifungal treatment, but the incidence of relapse is high (Budtz-Jorgensen and Bertram, 1970b; Budtz-Jorgensen, 1974; Cross et al., 2004). The existing conventional methods of
delivering drugs into intra-oral sites (such as topical application of miconazole gel, using nystatin suspension, chlorhexidine mouth wash) for the treatment of conditions of oral mucosa are inefficient (Budtz-Jørgensen, 1990a; Ellepola and Samaranayake, 2001). This is mainly due to the washing effect of saliva and oral musculature, which reduces the availability of the drug below the optimal therapeutic concentration (Ellepola and Samaranayake, 2001; Samaranayake et al., 2009). Also, a satisfactory outcome depends on patient compliance as frequent dose application is required which may lead to sub-optimal dosing (Samaranayake et al., 2009).

Dentists are frequently the first medical professionals to examine patients who have oral mucosal changes (Khatibi et al., 2011). Consequently, they are responsible for recognising the signs of disease, transforming them into a diagnosis, and planning the therapy, so it is important that the examination is carried out by a clinician who has adequate medical knowledge (Budtz-Jørgensen, 1981). Unfortunately, 30 % of doctors prescribe nystatin for oral candidosis without oral examination and only 9 % of doctors are aware that wearing a denture is a risk factor for oral candidal infection. This can result in recurrent candidal infection as a result of incorrect diagnosis or not considering important risk factors (Morgan et al., 2001).
1.2 Oral candidosis

1.2.1 Definition and description

Oral candidosis comprises a group of diseases that are associated with candidal infection. Furthermore it is by far the most common human fungal infection and manifests in a variety of clinical presentations (McIntyre, 2001; Akpan and Morgan, 2002). Oral candidosis may range from localised infections to acute systemic invasive diseases, both immunocompetent and immunocompromised individuals can be affected (McIntyre, 2001). In addition, it may present as a secondary infection superimposed on another medical condition (Budtz-Jorgensen et al., 1975; Akpan and Morgan, 2002). It can be a marker of immunosuppression and is therefore often referred to as “disease of the diseased” (McIntyre, 2001). Treatment of fungal infections necessitates removing or alleviating the predisposing condition (Rautemaa and Ramage, 2011). Importantly, the rate of opportunistic candidal infection has increased markedly among hospitalised patients (Fisher-Hoch and Hutwagner, 1995): this increase in the incidence is due to the increasing number of immunosuppressed patients and is associated with a high overall mortality rate (Fraser et al., 1992; Andes et al., 2009; Lopez-Martínez, 2010). Oral candidosis is a superficial infection; however, if treated ineffectively in immunocompromised patients, it may lead to invasive systemic infection and consequently increase the mortality rate (Gautam et al., 2010).
1.2.2 Organisms involved in oral candidosis

Oral candidal species are part of the normal oral flora in 18% of the normal healthy population (Cannon and Chaffin, 1999), with *C. albicans* being the most dominant species (Akpan and Morgan, 2002; Coco *et al.*, 2008). However, large variations are found in relation to the age, general health and dental health of the population studied (Akpan and Morgan, 2002). *Candida* species are opportunistic pathogens which cause disease when there is a disturbance in host-commensal balance (Ghannoum *et al.*, 2010; Rautemaa and Ramage, 2011). The change from commensalism to parasitism is most probably due to alteration in the oral environment of the host rather than the alteration of the yeast itself (Budtz-Jorgensen, 1974; Akpan and Morgan, 2002; Ghannoum *et al.*, 2010). Opportunistic pathogens incite disease in hosts whose local or systemic immune attributes have been impaired and the defence mechanisms have been damaged or innately dysfunctional (Nater *et al.*, 1978; Ghannoum *et al.*, 2010). Casadevall and Pirofiski (2003) showed that host-microorganism interaction determines the microbial pathogenesis outcome and the relevant host damage using damage-response curve (Figure 1.1).
Commonly a mixture of *Candida* species can be isolated from oral candidal lesions, *C. albicans*, *C. glabrata* and *C. tropicalis* have been isolated from these lesions (Budtz-Jorgensen *et al.*, 1975; Akpan and Morgan, 2002) (Table 1.1). *C. albicans* has a main role in the development of denture-induced candidosis (Cawson, 1963; Olsen, 1974; Akpan and Morgan, 2002; Coco *et al.*, 2008) but other species including *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. dubliniensis*, *C. tropicalis*, *C. kefyr* and *C. guilliermondii* have been increasingly isolated in immunocompromised patients (Sullivan *et al.*, 1995; Rautemaa *et al.*, 2006). Repeated and prolonged azole antifungal treatment has been related to the presence of these non-*albicans Candida* species (Cuéllar-Cruz *et al.*, 2012), which are resistant to commonly used azole antifungal agents and are considered second or third most common cause of systemic
candidosis (Delgado et al., 2009; Cuéllar-Cruz et al., 2012). Other studies have suggested that bacteria may also play a role in the disease (Budtz-Jorgensen and Theilade, 1983; Koopmans et al., 1988).

Table 1.1  The principal fungi that may infect the oral cavity (Akpan and Morgan, 2002)

<table>
<thead>
<tr>
<th>Candida species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td><em>Candida glabrata</em></td>
</tr>
<tr>
<td><em>Candida tropicals</em></td>
</tr>
<tr>
<td><em>Candida krusei</em></td>
</tr>
<tr>
<td><em>Candida guilliermondii</em></td>
</tr>
<tr>
<td><em>Candida parapsilosis</em></td>
</tr>
<tr>
<td><em>Candida dubliniensis</em></td>
</tr>
</tbody>
</table>

1.2.3 Predisposing factors

It is generally known that candidal infection does not affect healthy immunocompetent individuals (Cannon and Chaffin, 1999; McIntyre, 2001). Therefore, the presence of predisposing factors, breaching the immune defence mechanisms either at local or systemic level is essential for the development of the infection (Akpan and Morgan, 2002). A number of predisposing factors may result in the development of oral candidosis; all these factors affect the host-commensal balance, allowing the proliferation of the candidal organisms (McIntyre, 2001). These factors may be short-term or long-term in nature; this nature has significant impact
on the duration and the prognosis of the infection (these factors and others are discussed in more details in § 1.3.3).

### 1.2.3.1 Local factors

Short-term local predisposing factors include poor oral hygiene and topical steroid administration that can disturb the normal balance of oral flora and result in dissemination of the infection (Ritchie et al., 1969; Budtz-Jorgensen, 1990a; Rautemaa and Ramage, 2011). Dietary factors play important role in the oral homeostasis (Ritchie et al., 1969). A high carbohydrate intake provides a good source of nourishment for *Candida* (Ritchie et al., 1969).

Long-term local predisposing factors include mechanical irritation from dentures or any prosthesis or faulty occlusion may result in breakdown of the integrity of the oral mucosa and provide a good opportunity for the *Candida* to affect the injured tissues (Figueiral et al., 2007). In addition, xerostomia results in reduced salivary flow and predisposes the oral tissues to candidal infection (Ettinger, 1996). Local mucosal lesions (such as lichen planus) and smoking are also considered predisposing factors (Zeng et al., 2009; Rautemaa and Ramage, 2011). Dental problems such as periodontal pocketing, gingivitis and retained roots have been related to increased carriage of *Candida* leading to infection (Wang et al., 2006). Radiotherapy following malignancy is associated with an increased risk of oral candidosis (Dorko et al., 2001) because it has a direct negative effect on the rate of cellular turnover in the oral mucous membrane and the salivary flow (Ettinger, 1996; Deng et al., 2010).


1.2.3.2 Systemic factors

Use of antibiotics (especially broad spectrum) is considered a short-term systemic factor that can alter the normal balance of oral flora and result in spreading of infection (Ritchie et al., 1969; Budtz-Jorgensen, 1990a; Soysa et al., 2008). Humoral and cell-mediated immunity deficiencies (e.g. diabetes mellitus, AIDS) have been identified as a long-term systemic predisposing factor (Dorocka-Bobkowska et al., 1996; Akpan and Morgan, 2002). It has been estimated that 90% of AIDS patients develop oral candidosis (Akpan and Morgan, 2002). Other well known systemic factors include malignancy, malnutrition and physiological factors such as extreme ages and pregnancy (McIntyre, 2001): these factors are associated with imbalance in host-commensal relationship leading to infection. Deficiency states (iron, vit. B12, folate) are also associated with a lower level of host response (Ritchie et al., 1969; Rautemaa and Ramage, 2011), and gastro-oesophageal reflux disease could be a predisposing factor because it provides a favourable acidic growth environment for Candida species (Prusiski et al., 2002). All predisposing factors are summarised in Table 1.2.
Table 1.2 Predisposing factors in oral candidosis (Siikala, 2011)

<table>
<thead>
<tr>
<th>Local predisposing factors</th>
<th>Systemic predisposing factors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Short-term</strong></td>
<td><strong>Long-term</strong></td>
</tr>
<tr>
<td>Poor oral hygiene</td>
<td>Trauma (ill-fitting denture)</td>
</tr>
<tr>
<td>Topical steroids</td>
<td>Smoking</td>
</tr>
<tr>
<td>Dietary factors (high carbohydrate intake)</td>
<td>Radiation</td>
</tr>
<tr>
<td></td>
<td>Xerostomia</td>
</tr>
<tr>
<td></td>
<td>Local mucosal lesions and dental problems</td>
</tr>
<tr>
<td><strong>Short-term</strong></td>
<td>Broad-spectrum antibiotics</td>
</tr>
<tr>
<td></td>
<td>Dietary factors (deficiency state)</td>
</tr>
<tr>
<td><strong>Long-term</strong></td>
<td>Immune defect (AIDS)</td>
</tr>
<tr>
<td></td>
<td>Malignancy (leukemia)</td>
</tr>
<tr>
<td></td>
<td>Endocrine (diabetes mellitus)</td>
</tr>
</tbody>
</table>

1.2.4 Clinical presentation of oral candidosis

Many classifications have been suggested to describe the clinical forms of oral candidosis, the first classification of oral candidosis was proposed by Lehner (1966). He defined two major subdivisions:

- Acute, including pseudomembranous and atrophic candidosis.
- Chronic, including atrophic (Denture sore mouth) and hyperplastic candidosis.

The currently accepted classifications are presented in Table 1.3. Another common classification has been used, in which the lesions are divided into three main categories of acute, chronic, and mucocutaneous (Lynch, 1994). Acute candidosis is further subdivided into pseudomembranous and atrophic forms, and chronic
candidosis subdivided into atrophic and hyperplastic forms. Mucocutaneous candidosis can be presented as localised, familial, or syndrome related (Lynch, 1994).

Table 1.3 Classification of different clinical presentations of oral candidosis (Holmstrup and Axell, 1990; Samaranayake, 1991)

<table>
<thead>
<tr>
<th>Acute</th>
<th>Chronic</th>
<th>Candida-associated lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomembranous Erythematous</td>
<td>Hyperplastic</td>
<td>Denture-induced stomatitis</td>
</tr>
<tr>
<td></td>
<td>- Nodular</td>
<td>Angular cheilitis</td>
</tr>
<tr>
<td></td>
<td>- Plaque-like</td>
<td>Median rhomboid glossitis</td>
</tr>
<tr>
<td></td>
<td>Erythematous</td>
<td></td>
</tr>
</tbody>
</table>

1.2.4.1 Pseudomembranous candidosis

Pseudomembranous candidosis (or thrush) is characteristically an acute infection, even though many lesions are chronic in nature. The clinical presentation for this lesion is very characteristic; non-adherent creamy white patches, easily wiped with gauze to leave an underlying erythematous mucosa (McIntyre, 2001). Apart from neonates, who have no immunity to Candida species, thrush indicates an immunosuppression status or a local disturbance in oral flora, such as that caused by xerostomia, antibiotic treatment or corticosteroids. It is a feature in many immune defects, especially leukaemia and HIV disease. It can be an early feature of HIV infection (Budtz-Jorgensen, 1990a; Samaranayake, 1992). Thrush can affect any oral site, typically the palate or upper buccal vestibule posteriorly. Confirmation may be obtained by a smear biopsy.
1.2.4.2 Erythematous candidosis

Erythematous candidosis (previously known as antibiotic sore mouth) is associated with corticosteroids and broad-spectrum antibiotics. Erythematous candidosis resulting from the prescription of broad-spectrum antibiotics is the only oral candidosis where pain is a common symptom (McIntyre, 2001). Clinically, erythematous areas are seen on the dorsum of the tongue, palate or buccal mucosa and it can be associated with angular stomatitis.

1.2.4.3 Hyperplastic candidosis

Hyperplastic candidosis (also known as candidal leukoplakia) is a chronic white raised discrete lesion. Hyperplastic candidosis is a premalignant condition and it is indistinguishable from leukoplakia due to other causes. Therefore, biopsy is important in the management of this condition. The lesion may present as a bilateral homogenous or speckled lesion, which does not rub off. It usually affects commissural areas or rarely near the surface of the tongue (McIntyre, 2001).

1.2.4.4 Denture-induced candidosis

Denture-induced candidosis (or denture-induced stomatitis, or chronic atrophic candidosis, or denture sore mouth) is the most common clinical presentation of oral candidosis (Budtz-Jorgensen et al., 1975; Akpan and Morgan, 2002), and is usually associated with wearing prosthesis but many other factors are associated (Ritchie et al., 1969; Samaranayake et al., 2009; Jainkittivong et al., 2010). Typically, the lesions appear as an asymptomatic, erythematous mucositis limited to the denture-bearing mucosa (Budtz-Jorgensen, 1974; Figueiral et al., 2007).
1.3 Denture-induced candidosis

1.3.1 Definition and characteristics

Denture-induced candidosis is a term used to describe certain pathologic changes in the oral mucosa of denture-bearing tissues (Budtz-Jorgensen, 1974, 1981). It presents as a chronic bright diffuse erythema limited to the denture-bearing area in both jaws, but more frequently in the maxilla (Budtz-Jorgensen, 1981), as the upper dentures are in close contact with the mucosa and tend to be more conducive to the accumulation of plaque on the fitting surface. Denture-induced candidosis is a common inflammatory condition with high prevalence in denture wearing population (Budtz-Jorgensen et al., 1975; Figueiral et al., 2007). It has been documented that denture-induced candidosis affects 45-70 % of denture wearers (Figueiral et al., 2007). Women are the most commonly affected (Cawson, 1963; Arendorf and Walker, 1980; Figueiral et al., 2007).

The condition is usually symptomless apart from occasional soreness if complicated by thrush (Budtz-Jorgensen, 1974), consequently “Denture-induced candidosis” and “Chronic atrophic candidosis” have been suggested as more appropriate descriptions of the condition than “Denture sore mouth” as soreness is seldom a complaint (Newton, 1962). However, in association with denture-induced candidosis patients may suffer angular stomatitis (Cawson, 1963) and/or median rhomboid glossitis (Lynch, 1994).
1.3.2 Classification of denture-induced candidosis

Denture-induced candidosis can be manifested in a variety of clinical forms and can be categorised clinically according to Newton’s classification (Newton, 1962), which classified this condition into three categories:

- **Type 1**, characterised by pin-point hyperaemia. It shows small areas of inflammation in an otherwise normal tissue, which is usually found around the orifices of the ducts of the palatal mucous glands. It is the most common type (Kulak-Ozkan et al., 2002; Figueiral et al., 2007).
- **Type 2**, characterised by diffuse hyperaemia. A generalised inflammation of the entire denture-bearing area can be detected. The surface of the mucosa is smooth and slight trauma may be sufficient to cause bleeding.
- **Type 3**, presented as hyperplastic nodular reaction of the palatal mucosa which may be present over the entire denture-bearing area but is more commonly restricted to the central areas and is particularly found under relief areas or suction discs.

1.3.3 Aetiology factors of denture-induced candidosis

Denture-induced candidosis is a multifactorial condition and no primary aetiological cause has been identified (Ritchie et al., 1969; Figueiral et al., 2007). However, various factors are thought to be particularly significant:

- denture factors including continuous denture wearing and lack of hygiene (including reaction to denture plaque).
- infective factors.
- allergic reactions to denture base materials.
• dietary factors (including resultant haematological deficiencies).

• systemic factors and deterioration in the general state of health (including predisposing factors).

• antibiotic therapy.

• miscellaneous factors.

1.3.3 Denture factors

Many factors related to the prosthesis have been identified as risk factors for the development of denture-induced candidosis. These factors include trauma caused by ill-fitting dentures, unsatisfactory occlusion and articulation, and lack of oral and denture hygiene. There is good evidence that Newton's type I denture-induced candidosis is caused by trauma alone (Budtz-Jorgensen and Bertram, 1970a). It is assumed that pin-point hyperaemia in the palate (type I) is due to occlusion of the salivary ducts by a close fitting denture (Newton, 1962). In a study comparing denture adjustment versus antifungal therapy, lesions healed by proper adjustment of the dentures while antifungal therapy was ineffective (Budtz-Jorgensen and Bertram, 1970b). Several studies have provided evidence that denture-induced candidosis is present more frequently in patients with poor fitting dentures with a non-balanced occlusion (Figueiral et al., 2007; Emami et al., 2008). Continuous denture wearing has been suggested to cause denture-induced candidosis due to local injury (Nyquist, 1953), but it might also expose the mucosa to denture plaque for a longer time or alter the composition of this plaque (Kulak-Ozkan et al., 2002).

Poor oral and denture hygiene is frequently identified as a local aetiological factor (Fisher and Rashid, 1952; Budtz-Jorgensen and Bertram, 1970a; Rautemaa and Ramage, 2011). Bergendal (1982) found that patients with denture-induced
candidosis have more maxillary denture plaque than those with healthy mucosa. It has been found that an improvement in the level of oral and denture hygiene using mechanical or chemical means results in significant reduction in the number of patients with candidosis (Budtz-Jorgensen, 1979; Grimoud et al., 2005). These findings indicate that poor oral and denture hygiene is an important predisposing condition for denture-induced candidosis.

1.3.3.2 Infective factors

The infective causes including Candida and some bacterial genera have been reported to play an important role in denture-induced candidosis (Budtz-Jorgensen et al., 1983; Akpan and Morgan, 2002), but Candida species, mainly C. albicans are the ones most frequently involved (Davenport, 1970; Budtz-Jorgensen and Bertram, 1970a; Coco et al., 2008). Cahn (1936) was the first to report the presence of Candida in denture-induced stomatitis lesions. The causal relationship has subsequently been supported by mycological and immunological studies (Budtz-Jorgensen and Bertram, 1970a; Coco et al., 2008). The infection is primarily due to an overgrowth and colonisation of Candida species under the fitting surface of dentures (Budtz-Jorgensen and Bertram, 1970a). Budtz-Jorgensen and Bertram (1970b) found that the localised simple inflammation of the maxillary denture-bearing mucosa (type I) was caused by trauma, whereas the generalised simple (type II) and the granular inflammation (type III) more readily could be due to candidal infection, as hyphal structures were isolated in palatal smears consistently only among type II and III patients, although trauma may be a significant predisposing factor (Budtz-Jorgensen and Bertram, 1970a).
1.3.3.3 Allergy

Rattner (1936) was the first to report allergic reactions to denture base material. Further case reports reinforcing this finding have been presented (e.g. Bradford, 1948; Crissey, 1965; Barclay et al., 1999). Fisher (1956) has found that the incidence of these reactions is low and allergy to denture base materials rarely occurs. Nyquist (1952) in his study was not able to find cases of allergic reaction in 248 patients with denture-induced candidosis. Furthermore, a significant release of acrylic monomer will take place in new dentures only, and will be temporary. This fact rules out the irritating effect of residual monomer as a cause of denture-induced candidosis (Smith and Bains, 1956). In addition, the diagnosis is difficult to establish and cooperation is needed between the dermatologists and the dentists to diagnose these lesions (Kaaber et al., 1979).

1.3.3.4 Dietary factors and hormone deficiency

A diverse array of nutritional factors including high carbohydrate intake, iron deficiency, folic acid and vitamins deficiency have been identified as contributory factors of oral candidal infection (Shuttleworth and Gibbs, 1960; Ritchie et al., 1969; Samaranayake, 1986; Paillaud et al., 2004). Therefore it is not surprising that denture-induced candidosis patients have been found to be suffering from nutritional deficiencies (Budtz-Jorgensen, 1978). Iron deficiency has been reported to be an important factor to increase the susceptibility to Candida infection (Rose, 1968) and a higher incidence of folate deficiency in denture-induced candidosis patients has been reported than in a control groups (Samaranayake and McFarlane, 1981). Nutritional deficiencies such as amino acids, iron and certain vitamins of the B complex are believed to reduce the resistance of the oral mucosa (Samaranayake, 1986). It has
been shown that dietary supplements of proteins and minerals increase tolerance to dentures and resolve the inflammation (Deeley, 1965).

1.3.3.5 Systemic factors

A number of systemic diseases and treatment with various drugs may increase the susceptibility to oral candidosis (Knight and Fletcher, 1971; Akpan and Morgan, 2002). These factors include endocrine disturbances (diabetes mellitus) (Dorocka-Bobkowska et al., 1996; Dorko et al., 2001), immunodeficiency states (Samaranayake, 1992), nutritional deficiencies (iron deficiency, high carbohydrate intake), malignant diseases (leukemia) (Deng et al., 2010), and drugs such as antibiotics, and steroids (Knight and Fletcher, 1971; Budtz-Jorgensen, 1975). In these patients symptoms in association with denture-induced candidosis are usually pronounced (Ettinger, 1996). A wide range of other systemic diseases also play a secondary role due to the large number of therapeutic drugs that cause xerostomia, which is itself a predisposing factor (Ettinger, 1996).

1.3.3.6 Antibiotic therapy

It is well established that antibiotics increase susceptibility to infection with *C. albicans* (Ritchie et al., 1969; Soysa et al., 2008; Rautemaa and Ramage, 2011). Administration of oral antibiotics will disturb the oral flora by allowing an overgrowth of resistant organisms (Ritchie et al., 1969). Knight and Fletcher (1971) suggested that antibiotics stimulate the growth of *C. albicans* by killing nutrient-competiting bacteria, and hence an adequate amount of glucose would be available for the more slowly growing *Candida* species. It has been documented that prolonged antibiotic therapy in some medical problems (such as chronic urinary tract infection) increases the risk of development of denture-induced candidosis (Bergendal, 1982).
1.3.3.7 Miscellaneous factors

Psychological problems were detected in the majority of patients with denture-induced candidosis sampled by Nater et al (1978) using a personality questionnaire. Self-prosthetic treatment with home relining materials can also contribute to denture-induced candidosis (Welker, 1977).

1.3.4 Diagnosis of denture-induced candidosis

*C. albicans* is by far the most commonly isolated *Candida* species in denture-induced candidosis (Coco et al., 2008). However, non-*albicans* *Candida* species have become increasingly associated with oral candidosis especially in immunocompromised patients (high-risk) (Bagg et al., 2003; Coco et al., 2008). This highlights the importance of using accurate microbiological diagnostic methods (Rautemaa and Ramage, 2011). Quantitative estimates of the overgrowth of yeasts on the mucosa and the fitting surface of the denture either by culture or by microscopy of oral smears is needed to establish the diagnosis (Budtz-Jorgensen, 1974). Conventional sampling methods for oral *Candida* are swabbing, or scraping of the tissues, suction of saliva, or rinsing the mouth. The most valuable tools in the diagnosis of oral candidosis are semi-quantitative cultures and oral smears (Budtz-Jorgensen, 1990b). Biopsy (a microscopic examination of tissue sections) is required if premalignant or malignant changes of the epithelium are suspected. Since agglutinating, haemagglutinating, precipitating, and immunofluorescent antibodies are found in people without signs of *Candida* infection, serologic tests for *Candida* are not diagnostic methods, and the raise in the antibody response in patients with oral candidosis is only moderate. However, such tests may be a prognostic instrument and a valuable
tool in patients with severe oral candidosis who respond poorly to antifungal therapy (Budtz-Jorgensen, 1990b).

For the collected samples to be representative, it is important to sample an adjacent non-renewing surface in addition to the affected area (Rautemaa et al., 2006). Oral rinsing is a helpful mean to indicate the intra-oral fungal load (McIntyre, 2001; Coco et al., 2008). Direct sonication of the denture is a valuable method which remarkably improves both quantification and diagnosis of candidal infection (Coco et al., 2008). Haematological investigations are also important to assess any underlying predisposing factors such as deficiency of iron, vitamin B12 or folate (McIntyre, 2001). In patients with a localised simple type of denture-induced candidosis, microbiological examinations are not essential as these lesions do not appear to be caused by C. albicans (Budtz-Jorgensen, 1974). In patients with recurrent candidal infection or high-risk patients, species level identification using different standard biochemical methods and susceptibility testing is crucial. These tests are important to identify if resistant species are involved which is important to decide an appropriate antifungal treatment (Rautemaa and Ramage, 2011).
1.4 *Candida albicans*

1.4.1 Definition and characteristics

*Candida albicans* is part of the normal flora of the oral cavity but is present in small numbers in healthy individuals (Cannon and Chaffin, 1999). However, it is regarded as the principal aetiological factor in denture-induced candidosis (Cawson, 1963; Budtz-Jorgensen, 1974; Coco et al., 2008). *Candida* has been isolated from over 80 % of patients suffering from denture-induced candidosis (Figueiral et al., 2007). The increasing incidence of infections caused by *C. albicans* highlights the complexity of diagnosing and treating candidosis in the immunocompromised patient (Fisher-Hoch and Hutwagner, 1995; Rautemaa and Ramage, 2011). Overcoming these challenges requires a good understanding of host-candidal interactions. An important characteristic of these interactions is the virulence attributes of the fungus (Cutler, 1991).

*Candida* is a eukaryotic organism with a cell wall that lies external to the plasma membrane. There is a nuclear pore complex within the nuclear membrane. The plasma membrane contains large quantities of sterols, usually ergosterol (McCullough et al., 1996). *C. albicans* is an obligate associate of human beings with a dimorphic nature (Calderone and Braun, 1991). It is dimorphic because it has two forms, it can proliferate into either a yeast form or a hyphal form. Switching between these two forms depends on a complex interaction of external and internal factors (Figure 1.2) (Calderone and Braun, 1991). However, yeast and hyphal cells are not the only morphological states of *C. albicans*. Other morphological states can form in response to certain environmental and genetic conditions for example,
the opaque form required for mating, the pseudohyphal cell, and the chlamydospore (Whiteway and Bachewich, 2007).

Figure 1.2 Dimorphic nature in *Candida*: Blastospore form (a), Hyphal form (b)

*Candida* multiplies by mitotic cell division or budding. When the bud has grown to an optimal size, nuclear division occurs and a septum is formed between the two cells. Multiple cells divided by septa will form hyphae (Webb *et al.*, 1998b). It forms soft cream-coloured colonies when grown under aerobic conditions. Growth is usually detected within 48-72 h; the ability of yeasts to grow at 37 °C is an important characteristic to be considered in their identification from clinical specimens (Webb *et al.*, 1998b).

The most densely site populated with *C. albicans* is the dorsum of the tongue. Other oral sites such as mucosa, palate and plaque-covered tooth surfaces are colonised secondarily (Arendorf and Walker, 1980). It has also been shown that the labial vestibular sulcus is the most important primary reservoir for *Candida* and especially
C. albicans (Rautemaa et al., 2006). In denture wearers, the denture fitting surface is the primary and the most important source of C. albicans (Coco et al., 2008).

1.4.2 The virulence mechanisms and the factors affecting distribution of Candida in the oral cavity

Fungal virulence and pathogenicity depends on specific virulence factors and mechanisms that have the capability to breach the host defence mechanisms (van Burik and Magee, 2001). Identification of virulence factors has received particular attention, this interest based on the fact that these factors contribute to the pathogenicity of the organism (McCullough et al., 1996; van Burik and Magee, 2001). The identification of these factors is highly important for the development of effective therapeutic strategies (Dhir et al., 2007).

1.4.2.1 Adherence

Candida can resist the clearing mechanisms of the host by adherence to a target surfaces. Adherence is a very important prerequisite in the pathogenesis of oral candidal infections (McCourtie and Douglas, 1984; Hoffman and Haidaris, 1993; He et al., 2006). It involves two phases: the initial adherence is not specific and results from the free-surface energies of the surfaces and the Candida. The second phase of adhesion is more specific and it involves particular adhesion-receptor interactions. The specific interaction between Candida and a host requires interaction between Candida cell ligands and host cell receptors (Calderone and Braun, 1991). The protein portion of the mannanproteins adhesion of Candida interacts with N-acetylglucosamine containing surface glycoproteins of epithelial cells (Calderone and Braun, 1991). This stage is necessary for the tight binding of the yeast
to a specific substrate that facilitates colonisation and invasion (Samaranayake et al., 1980).

There are also other factors affecting the adherence of yeast to surfaces, including the type of material (Okita et al., 1991), surface roughness (Radford et al., 1998) and consumption of a carbohydrate-rich diet (cultivation media) (Samaranayake et al., 1980). In addition, the morphologic form of Candida has an effect; hyphal forms adhere more significantly than that of the blastospore forms (Samaranayake and MacFarlane, 1982).

I.4.2.2 Saliva

It has been reported that saliva reduces the adhesion of C. albicans to acrylic; however, serum enhances its adhesion (Samaranayake et al., 1980; Rautemaa and Ramage, 2011). It has been shown that preconditioning of biomaterials with serum enhances biofilm formation (Frade and Arthington-Skaggs, 2011) and this may explain the role of trauma as an important aetiological factor in denture-induced candidosis (Nyquist, 1952). Another study suggested that mannanproteins on the surface of C. albicans have receptors in salivary proteins or mucin (Nikawa and Hamada, 1990), and mucin-containing saliva promotes adhesion of Candida to acrylic dentures (Hoffman and Haidaris, 1993). Mixed saliva has been found to reduce the in vitro adherence of C. albicans (Samaranayake et al., 1980). These results support the work of Olsen and Haanaes (1977), who found increased yeast colonisation of upper acrylic plates in monkeys with reduced salivary flow. Reduced salivary flow rate and reduced secretion of antimicrobial proteins into saliva are known risk factors for oral candidal infections (Ueta et al., 2000).
1.4.2.3 Growth at different pHs

A low pH environment favours Candida growth and colonisation (Webb et al., 1998b; Sherman et al., 2002); however, C. albicans can grow both in acidic and basic media and can adapt to environments of diverse pH (Buffo et al., 1984). This ability to tolerate a wide range of pH is an important virulence factor. C. albicans has two pH-regulated genes, PHR2 and PHR1, the former expressed at acidic pH and the latter at neutral and basic pH (Muhlschlegel and Fonzi, 1997).

1.4.2.4 Nutritional and Metabolic factors

In order to thrive in the host, fungi need to be able to carry out biosynthetic and metabolic reactions. Experiments of auxotrophic mutants of C. albicans (auxotrophs require a nutrient that the host does not require) have pointed out that the inability to synthesise purines, pyrimidines, or haem significantly diminishes virulence and pathogenicity (Kirsch and Whitney, 1991).

1.4.2.5 Necrotic factors

Extracellular proteinase has been identified as a potential necrotic factor (Staib, 1969). The production of such enzymatic necrotic factor results in a breach of the structural barriers of host cells during infection. C. albicans is known to secrete phospholipases, which have a role in pathogenicity, and isolates associated with high mortality rates in mice have the highest phospholipase activity (Ibrahim et al., 1995).

1.4.2.6 Mannanoproteins (cell surface polysaccharide)

It has been established that the cell wall of C. albicans is composed mainly of mannanoproteins and glucans and a small amount of chitin (Bishop et al., 1960). These mannanoproteins have an important role in the interaction between Candida
and host cell receptors, which is mainly located in the outer layer of the yeast cell wall (Calderone and Braun, 1991).

1.4.2.7 Cell surface hydrophobicity

Changes in hydrophobicity and hydrophilicity of *C. albicans* are thought to be due to changes in the external cell wall protein (Hazen *et al.*, 1990). It has been shown that hydrophobic *C. albicans* cells can bind abundantly and diffusely to host tissues free of macrophages, while hydrophilic cells bind to regions with macrophages (Hazen *et al.*, 1991). This results in removing of hydrophilic cells by phagocytosis but not hydrophobic cells, which can then flourish and colonise epithelial surfaces. It has been postulated that London-Van der Waals forces (hydrophobic molecular forces) are responsible for adhesion of *Candida* to plastic surfaces. In addition, it has been documented that the adhesion of *Candida* spp. to hydrophobic surfaces is more rapid than to hydrophilic ones (Frade and Arthington-Skaggs, 2011). The ability of *Candida* to adhere to polymeric surfaces may give it direct access to human hosts (Klotz *et al.*, 1985; He *et al.*, 2006).

1.4.2.8 Oral bacteria

The presence of bacteria is a contributing factor in the colonisation of *Candida* strains in the oral cavity and plays a complex role in regulating the adhesion of *Candida* (Samaranayake *et al.*, 1980). It has been shown that adhesion of *C. albicans* to acrylic surfaces is enhanced when the yeast is incubated simultaneously with *Streptococcus mutans* (Branting *et al.*, 1989), as well as with *Streptococcus salivarius* (Samaranayake *et al.*, 1980).
1.4.2.9 **Multiple cell forms**

Dimorphic fungi regulate their cellular morphology in response to environmental conditions. For example, blastospore form of *C. albicans* prevails in rich media, whereas hyphae which composed of elongated cells attached end-to-end form in response to starvation, serum, and other conditions (Land *et al.*, 1975). A variety of environmental changes, including a shift from an aerobic to a fermentative metabolism or growth on particular compounds such as N-acetyl glucosamine, cause *C. albicans* to switch from yeast to filamentous growth. This switching is accompanied by changes in carbohydrate metabolism and an interruption of electron transfer within the cell (Land *et al.*, 1975). Temperature and pH can both regulate *C. albicans* dimorphism (Land *et al.*, 1975).

The hyphal form of *C. albicans* is associated with its invasiveness (Kimura and Pearsall, 1980; Buffo *et al.*, 1984; Calderone and Braun, 1991). In addition, there is a strong relation between germ tube formation and increased adhesion of *C. albicans* to buccal epithelial cells, the hyphae of *Candida* enhance adhesion to human mucosal cells (Kimura and Pearsall, 1980).

1.4.2.10 **Phenotypicity**

Some *C. albicans* strains are capable of undergoing phenotypic changes (regulation of expression of gene activity without alteration of genetic structure), which result in a change in colonial morphology. This can be advantageous to the yeast, particularly in relation to being able to tolerate antifungal therapy (McCullough *et al.*, 1996). An example of phenotypicity is white-opaque phenotypes. White colonies almost exclusively contain classical yeast cells whereas opaque colonies contain bean-shaped cells and differ from white cells in a variety of ways, including surface properties,
gene expression, temperature sensitivity and pathogenicity (Kennedy et al., 1988). White cells can switch to opaque cells within one generation, and can adhere more significantly to buccal epithelial cells (BEC) than the latter (Kennedy et al., 1988). The white phenotype is more virulent in a systemic mouse model of infection, whereas opaque cells are more effective in colonisation in a mouse cutaneous infection model (Kvaal et al., 1999).
1.5 Denture plaque biofilms

1.5.1 Definition

Denture plaque biofilm represents a protected community of microbial growth that allows survival in a hostile environment (Ramage et al., 2005). It is a structured community of microorganisms with increased cell density surrounded by a self-produced polymeric matrix that is adherent to an inert or living surface (Costerton et al., 1999). Ultrastructural studies have revealed that denture plaque is a thick material composed of cocci, rod-shaped bacteria, desquamated epithelial cells and yeasts (Radford and Radford, 1993). It is now known that the majority of microorganisms live in a biofilm community (Costerton et al., 1999), rather than planktonic lifestyle (free-floating).

1.5.2 Candida biofilms

Fungi, like bacteria, are also capable of forming biofilms (Ell, 1996; Chandra et al., 2001a; Ramage et al., 2005). *C. albicans* is by far the most common fungal species associated with biofilm formation (Douglas, 2002). Cells within biofilms have the ability to withstand host immune defences and antifungal therapy (Chandra et al., 2001a) and biofilm formation on medical and dental devices can cause device failure and provide a source for future continuing infections (Ell, 1996; Douglas, 2002). The detailed structure of *C. albicans* biofilms was first examined by scanning electron microscopy (SEM) (Hawser and Douglas, 1994). Figure 1.3 shows SEM image of a mature *C. albicans* biofilm. These biofilms are composed of yeast, hyphal, and pseudohyphal elements.
1.5.2.1 Formation and characterisation of *C. albicans* biofilms

*Candida* has an exceptional ability to adhere to artificial surfaces and prostheses (He et al., 2006). Initial attachment of yeast cells is followed, after 3 to 6 h, by germ tube formation. Fully mature biofilms, produced after incubation for 24 to 48 h, consist of a dense network of yeasts, hyphae and pseudohyphae (Hawser and Douglas, 1994). The initial focal attachment of individual cells to a substratum is closely followed by cell division and biofilm development. Mature *Candida* biofilms exhibit extensive spatial heterogeneity and form a complex three-dimensional structure (Chandra et al., 2001a). The biofilms are a potential source of fungal infection and need to be cleaned and interrupted thoroughly (Coco et al., 2008; Rautemaa and Ramage, 2011).
1.5.2.2 Role of morphogenesis in C. albicans biofilm formation

It has been demonstrated that hyphae are essential elements to provide the structural integrity of biofilms and give the multilayered complex structure of mature biofilms (Baillie and Douglas, 1999). It is possible that dimorphism has a role in the development of biofilms; however, Watamoto et al (2009) showed that mutants lacking the capability to form hyphae were resistant to azole antifungal agents. These findings may indicate that filamentation is not the major determinant of antifungal resistance in Candida biofilm.

1.5.2.3 Candida biofilms resistance

Resistance to antifungal agents is the insensitivity of microorganisms to that antifungal agent. In vitro, it is determined using minimum inhibitory concentrations (MIC) that is the lowest concentration of an antifungal agent required to inhibit the visible growth of microorganism (Clinical Laboratory Standard Institute, 2008a). The susceptibilities of Candida species to different antifungal agents vary significantly (Versalovic, 2011). Generally, C. albicans strains are susceptible to most commonly used antifungal agents (Versalovic, 2011). Candida species, such as C. glabrata and C. krusei, are inherently resistant to commonly used azoles (Coco et al., 2008; Delgado et al., 2009).

It has been shown that the structure of Candida biofilm is highly associated with a dramatic increase in levels of resistance to the most widely used antifungal agents (Ramage et al., 2002a), and biofilm cells are up to 1000 times more resistant to antimicrobial drugs than planktonic cells (Seneviratne et al., 2008). High MICs are commonly reported for polyenes and azole antifungal agents in Candida biofilms (Ramage et al., 2002b; Seneviratne et al., 2008). The nature of biofilm architecture,
altered gene expression and the physiological attributes of biofilm organisms give an intrinsic resistance to antimicrobial agents (Douglas, 2002). The mechanisms of biofilm resistance to antimicrobial agents are poorly understood and it is thought to be multifactorial (Ramage et al., 2002a).

This resistance is thought to be related to the following mechanisms (Figure 1.4):

- Delayed penetration of the antimicrobial agent through the biofilm matrix, because of the high density of cells within the biofilm and the matrix around the biofilm (Al-Fattani and Douglas, 2006; Seneviratne et al., 2008).
- Altered growth rate of biofilm organisms (Kumamoto, 2002). Biofilm cells grow significantly more slowly than planktonic cells. Consequently, the cells take up antimicrobial agents more gradually.
- Physiological changes due to the biofilm mode of growth (Gilbert et al., 1997) in relation to nutrient and interactions between matrices.
- The expression of resistance genes, particularly those encoding efflux pumps (Ramage et al., 2002a). This leads to upregulation of drug transporters leading to active export of the antimicrobial from the cell.
- Mutations in the genes encoding the drug receptors in the cell which leads to failure in the interaction (Lopez-Ribot et al., 1998).
- Upregulation of the gene encoding the drug receptors in the cell (Lopez-Ribot et al., 1998).
Figure 1.4 The different mechanisms of resistance in *C. albicans* (Siikala, 2011)
1.6 Management of denture-induced candidosis

The high incidence of oral candidosis appears to be due to the multiplicity of predisposing factors (which facilitates switching of the commensal presence of *Candida* to a pathogenic one), and the increasing number of immunosuppressed patients (Ellenpolo and Samaranayake, 2001; Lopez-Martínez, 2010), so it is important to manage these conditions effectively. Moreover, as oral candidosis is mainly a biofilm infection; effective treatment requires disruption of the biofilm (Rautema and Ramage, 2011). In addition, predisposing factors should be addressed to prevent the recurrence of fungal infections. Variable treatment protocols have been suggested for the treatment of oral candidosis (Samaranayake et al., 2009; Rautema and Ramage, 2011). Although denture-induced candidosis could be treated by methods targeted solely towards the oral mucosa, denture-induced candidosis is associated with a proliferation of *Candida* which is mainly within plaque on the denture rather than in the palatal mucosa (Davenport, 1970). It has therefore been suggested that treatment should be directed to the denture rather than the mucosa. Cracks within the denture represent a yeast reservoir, which are difficult to be removed by mechanical brushing (Ramage et al., 2004). For successful treatment it is essential to eradicate the yeast from both the oral tissues as well as from the denture base (Kulak et al., 1994; Ramage et al., 2004).
1.6.1 Treatment modalities directed toward oral mucosa

1.6.1.1 Antifungal agents

Fungi and the human host are both eukaryotes. Therefore, drugs that inhibit protein, RNA, and DNA synthesis are potentially toxic for the host and thus antimycotic agents have limited potential targets (White et al., 1998). The efficacy of antifungal treatment is well documented (Ellepola and Samaranayake, 2000a). A number of effective antifungal agents, which can be administered either topically or systemically, are available for management of oral candidosis (Graber, 1994). Four classes of antifungal compounds are currently available for clinical use, the polyenes, the azoles, the allylamines and the echinocandins. The first three classes target ergosterol synthesis (major sterol in fungal cell membrane), whereas the echinocandins target nucleic acid synthesis (Versalovic, 2011).

The first line treatment for oral candidosis should be topical in healthy patients with uncomplicated infection and no risk for systemic spread (Ellepola and Samaranayake, 2000a). Systemic treatment is indicated in complicated oral candidosis in immunocompromised patients in addition to topical treatment (Akpan and Morgan, 2002). Patients with repeated or prolonged courses of antifungals should be treated with antifungal agents that have low risk for development of resistant strains (Johnson et al., 1995). Consequently, the choice of the treatment depends on the complexity of the infection, the immunological status of the patient and the chronicity of the predisposing factors (Rautemaa and Ramage, 2011).
A) Polyene antifungal agents

Amphotericin B and nystatin, the first clinically available and effective antifungal agents, are common topical antifungal agents (Budtz-Jorgensen and Lombardi, 1996; Ellepola and Samaranayake, 2000a). Although significant resistance in yeasts has been reported in isolates from patients with prolonged neutropenia, fungal resistance to these agents is rare (Graber, 1994; White et al., 1998).

- **Amphotericin B**

Amphotericin was discovered in the 1950s (Donovick et al., 1956). It inhibits fungal growth through its interaction with ergosterol (the main sterol in fungal membrane). This interaction results in channel formation which, in turn, leads to loss of the membrane selective permeability, leakage of cellular components and cell death (Ellepola and Samaranayake, 2000a). Their selectivity is based upon the difference in sterol composition of the yeast and host cells; that is, these polyenes have a higher affinity for yeast ergosterol than for host cholesterol (Ellepola and Samaranayake, 2000a). Amphotericin can be administered topically and intravenously. It has a fungistatic or fungicidal effect depending on its concentration with broad-spectrum activity. Unfortunately one of the most common side effects of systemic amphotericin B is nephrotoxicity, although new lipid-associated formulations of amphotericin B have been introduced in an attempt to alleviate the agent toxicity (Versalovic, 2011).

- **Nystatin**

Nystatin is the most popular agent for treating superficial infections with *Candida albicans* (Cawson, 1963; Ellepola and Samaranayake, 1999; Versalovic, 2011), and it is an effective agent in type II and III candidal infections (Budtz-Jorgensen and Bertram, 1970b). It has a mode of action similar to that of amphotericin B
Amphotericin B and nystatin have unpleasant taste which results in less patient compliance (Martin et al., 1986). However, despite the proven in vitro efficacy of nystatin against *C. albicans*, treatment with this drug may fail particularly in patients with HIV/AIDS (Nualwan, 1976; Pons et al., 1997). It has been proven that nystatin is very effective antifungal agent and the explanation for the failure of therapy is poor patient compliance, especially with the gel form, although when provided in pastille form it effectively cures the infection (Martin et al., 1986).

**B) Azole antifungal agents**

Azoles are also commonly used for the management of oral candidosis. Azole-containing antifungals all interfere with sterol synthesis and are classified as ergosterol-biosynthesis inhibitors. They act by interfering with synthesis of fungal cell membranes by inhibiting cytochrome P-450 enzymes (14 α-demethylase) (gene ERG 11), which convert 14 α-methylsterols to ergosterol in the cell membrane (Versalovic, 2011). This blockage results in depletion of ergosterol and accumulation of 14 α-methylsterols that may account for reduction in *Candida* adhesion to buccal epithelial cells when exposed to fluconazole (Zepelin et al., 2002). The fungal cytochrome P-450 system is more sensitive to antifungal drugs than the cytochrome P-450 of humans. This fact explains the selective action of these drugs against fungi (Shepherd et al., 1985). Several resistance mechanisms have been reported, including upregulation of multidrug efflux transporter genes; upregulation of ERG11 gene that encodes 14 α-demethylase (Figure 1.4) (Pfaller et al., 2010).

- **Fluconazole**

Fluconazole efficacy in treating oral candidosis has been investigated and good clinical outcomes have been reported (Lumbreras et al., 1996; Ellepola and
Samaranayake, 2000a; Koray et al., 2005). Reduction in candidal adhesion to buccal epithelial cells (BEC) is observed after treatment with fluconazole. As discussed previously (§ 1.4.2.1), the adhesion of fungi to host mucosal surfaces is a prerequisite for colonisation and infection (Samaranayake and MacFarlane, 1982). Although fluconazole reduces the adhesion of C. albicans (Zepelin et al., 2002), resistance has been reported in HIV patients (Zepelin et al., 2002). Prolonged and repeated exposure to low-dose fluconazole is associated with resistance development (Johnson et al., 1995; Rautema and Ramage, 2011). Fluconazole is well tolerated and adverse effects are mild and subjective. They include nausea, headache, abdominal discomfort, diarrhoea and vomiting (Ellepola and Samaranayake, 2000a). Fluconazole should be mainly used in uncomplicated infections with high enough doses to reach effective therapeutic levels (Rautema and Ramage, 2011).

- **Itraconazole**

Itraconazole has broader spectrum activity compared to fluconazole. It is widely used in the treatment of superficial fungal infections as well as for systemic infections (Versalovic, 2011) and resistance is rarely documented. It is well tolerated with few side effects.

- **Miconazole**

Miconazole is a synthetic antifungal agent. It inhibits ergosterol synthesis and accumulates reactive oxygen species and peroxide molecules, leading to cell death (Laudenbach and Epstein, 2009). It exhibits several drug interactions and therefore it is not appropriate for patients using multiple medications but rather for healthy patients with uncomplicated infection. Development of resistance is a common finding with repeated use of miconazole (Rautema et al., 2008).
- **Clotrimazole**

Clotrimazole has a fungistatic effect. It binds to fungal cell membranes affecting permeability and leading to loss of vital intracellular elements (Laudenbach and Epstein, 2009). It is effective against superficial oral candidal infections. It is particularly useful in treating angular cheilitis due to its action against *Candida* and also *Staphylococci* (Ellepola and Samaranayake, 2000a).

- **Ketoconazole**

Ketoconazole has broad spectrum activity and was the first orally available azole (Ellepola and Samaranayake, 2000a). Its use is limited due to its hepatotoxic effects.

- **Posaconazole and Voriconazole**

Both posaconazole and voriconazole are second generation azoles with a broad spectrum of activity and higher efficiency compared to otherazole antifungal agents (Ellepola and Samaranayake, 2000a). They are well tolerated and resistance has not been reported (Versalovic, 2011). Their use should be restricted to immunocompromised patients and patients with refractory infections resistant to other antifungal agents because both agents require level monitoring and as a result they are more technically demanding and are also not cost effective (Versalovic, 2011).

Following brief exposure to antifungal agents, suppression of yeast growth persists, and this effect is termed post antifungal effect (PAFE) (Ellepola and Samaranayake, 1998). The main significance of this effect is the impact that it may have on the dosage regimens of therapeutic agents (Ellepola and Samaranayake, 1998). Antifungal agents inducing longer post antifungal effect can be administered with more spaced dosing.
intervals, with the same efficacy and with fewer adverse effects (Ellepola and Samaranayake, 1998). It has been shown in many studies that *Candida* biofilms are resistant to conventional antifungal treatments including amphotericin B, fluconazole, itraconazole and ketoconazole (Chandra *et al.*, 2001a; Chandra *et al.*, 2001b; Ramage *et al.*, 2002a; Ramage *et al.*, 2002b). Resistance to fluconazole is common in HIV patients (White *et al.*, 1998). The suggested mechanism, through which the resistance can result, is the increased efflux of the drug by pumps on the cell membrane that remove the antifungal drug out of the cell (Ramage *et al.*, 2002a). It has been reported that these biofilms are up to 4000 times more resistant to fluconazole when compared to planktonic cells (Hawser and Douglas, 1994). Figure 1.5 shows the mechanism of action of different antifungal agents.

**Figure 1.5 Mechanism of action of different antifungal agents**
C) Echinocandins

The demand for new effective therapies other than conventional ones has been developed. A vast array of new antifungal agents such as caspofungin, anidulafungin and micafungin are now available. These agents have shown activity against fluconazole-resistant *C. albicans* (Ramage et al., 2002b). Antibiofilm activity has been demonstrated with echinocandins *in vitro* (Ramage et al., 2002b; Ramage et al., 2011).

These agents are only available in intravenous formulations and they are very expensive, limiting their use to systemic and invasive candidal infections. The effect of combination of antifungal agents has been examined for example, antagonistic effect of high fluconazole doses with caspofungin was observed (Bachmann et al., 2003).
1.6.1.2 Antimicrobial agents

It has been shown that chlorhexidine gluconate has broad-spectrum antimicrobial activity including Candida (Giuliana et al., 1999; Ellepola and Samaranayake, 2001). It is a safe oral antiseptic with well documented antifungal and antibacterial activity in vitro and in vivo, as well as anti-plaque effects (Epstein, 1990; Lal et al., 1992). It has been shown to be an effective against C. albicans biofilms and is able to reduce the biofilm viability by 80 %-100 % (Lamfon et al., 2004; Ramage et al., 2011). Moreover, chlorhexidine shows superior efficacy compared toazole antifungal agents (Ellepola and Samaranayake, 2001; Ramage et al., 2011). Therefore, data support the use of chlorhexidine mouth rinses as an alternative or adjunct to conventional antifungals in the management of oral candidosis (Giuliana et al., 1997; Koray et al., 2005; Ramage et al., 2011).

Chlorhexidine has an equivalent or superior antifungal effect to that of fluconazole with fewer potential complications (Koray et al., 2005; Ramage et al., 2011). Conservative intervention has been advised to be employed by using oral mouth rinses to prevent the adverse effects of systemic drugs used for treatment of oral candidosis (Koray et al., 2005). Chlorhexidine in 0.2 % concentration mouth rinse has been successfully used in the treatment of denture-induced candidosis (Ellepola and Samaranayake, 2001), so it is effective at low concentrations. Chlorhexidine affects Candida by various mechanisms. It reduces the adherence of C. albicans to BEC (Jones et al., 1997). It has been demonstrated that chlorhexidine has the ability to suppress candidal hyphae formation (Ellepola and Samaranayake, 2000b). Another important factor that contributes in candidal pathogenicity is the
cell surface hydrophobicity of Candida (Hazen et al., 1991) which reduces following treatment with chlorhexidine (Anil et al., 2001).

The crucial feature of chlorhexidine is its substantivity in the oral cavity which prolongs its therapeutic effect (Bonesvoll et al., 1974); it binds to salivary pellicles as well as hard tissues in the oral cavity, resulting in chlorhexidine titres in saliva for 12 h or more after rinsing. About 30 % of chlorhexidine may be retained in the mouth for 24 h after 1 min rinsing (Bonesvoll et al., 1974). In addition, it has a post antifungal effect, which results in suppression of fungal growth after removal of the agent (Ellepola and Samaranayake, 2000b). Chlorhexidine has been used in combination with other antifungal agents. It has been indicated that treatment with fluconazole plus chlorhexidine has a synergistic effect (Kulak et al., 1994). Further, the incidence of oral candidosis was shown to decrease significantly when chlorhexidine was used as prophylactic agent in a group of neoplastic patients (Ferretti et al., 1990). Topical use of chlorhexidine may have some side effects. The most common ones include superficial staining of the teeth and other oral surfaces, an increase in calculus formation, and an alteration in taste perception (Helms et al., 1995; Gürgan et al., 2006) but these unwanted effects are reversible and well tolerated (Rushton, 1977). Conflicting data have been reported concerning cytotoxicity effect of chlorhexidine. In vitro studies have reported that chlorhexidine results in DNA damage in leukocytes, BEC and human alveolar cells at clinically relevant concentrations (Ribeiro et al., 2004; Cabral and Fernandes, 2007). However, clinical experience and in vivo studies have shown a beneficial healing effect in different mucosal and periodontal conditions (Brägger et al., 1994; Lang et al., 1994).
Other mouth rinses (such as cetylpyridinium chloride) have demonstrated in vitro antifungal properties; however more clinical trials are needed to evaluate efficiency in vivo (Giuliana et al., 1997). Aqueous garlic extract has also been shown to have inhibitory effects in vitro on the adhesion of Candida to BECs, in addition treatment of blastospores with the extract reduced the ability to form germ tubes (Ghannoum, 1990).

1.6.2 Treatment modalities directed toward the denture base

Although systemic dosage of antifungals may be effective against mucosal lesions, they offer no solution to a Candida infested denture fitting surface (Budtz-Jorgensen and Bertram, 1970b). Persistent fungal presence on the fitting surface of a denture often leads to cross infection and recurrence of the mucosal lesions (Nualwan, 1976). C. albicans has been found on both hard denture base acrylic resins and silicone-based resilient liner materials in vitro (Allison and Douglas, 1973; He et al., 2006). However, adhesion of C. albicans to denture base acrylics is less than to the silicone liners (Okita et al., 1991; Nevzatoglu et al., 2007).

1.6.2.1 Hygienic measures and denture cleansers

Poor denture hygiene is a predisposing factor to candidal infection and poor oral hygiene increases Candida colonisation (Grimoud et al., 2005; Coco et al., 2008). As a result, hygienic care of the fitting surface of the denture would seem to be an appropriate treatment strategy (Fisher and Rashid, 1952; Grimoud et al., 2005) and improvement of the oral hygiene is a basic management for oral candidal infections before imparking more invasive therapeutic options (Rautemaa and Ramage, 2011). However, denture biofilm control that uses only brushing is not as effective as chemical cleansing in removing denture plaque and also in preventing
denture-induced candidosis (Kulak et al., 1997), and cleaning with water alone is the least effective method compared to all other hygienic protocols (Harrison et al., 2004).

Mechanical cleaning of dentures is an effective mean to maintain good denture hygiene (Kulak et al., 1997). However patients usually find difficulty to maintain the denture hygiene, so it is crucial to use chemical cleansing agents for disinfection (Budtz-Jørgensen, 1979). Chemical cleansing is more effective in reducing prosthesis plaque and in preventing denture-induced candidosis in comparison with mechanical cleaning (Kulak et al., 1997). Denture cleansers have been studied to identify the ideal cleanser (Budtz-Jørgensen, 1979). The ideal denture cleansers should be easy to use, bactericidal and fungicidal, efficient in removing organic and inorganic deposits and safe to dentures (Neill, 1968).

Denture cleansers can be divided into two groups:

- **Paste type cleansers**: they contain abrasive agents which significantly roughen the denture base material, resulting in increased plaque accumulation (Harrison et al., 2004), but they provide an alternative to immersion cleaning for those patients who are unwilling to leave their dentures out at night (Neill, 1968). These products causes damage to acrylic resin and thus only those with minimum abrasiveness should be used (Neill, 1968).

- **Immersion type cleansers**: they are the most suitable for denture base material due to their low abrasiveness and high effectiveness in removing organic debris (Harrison et al., 2004), and accordingly less plaque retention.
This type mainly includes peroxide and sodium hypochlorite and it has been found to be effective against *C. albicans* (Rudd *et al.*, 1984).

Sodium hypochlorite is effective in removing plaque (Kulak *et al.*, 1997). Webb *et al.* (1995) reported that sodium hypochlorite in a concentration below the MIC reduces the adhesive abilities of *Candida* species. Therefore, it can be used as an effective antifungal denture soak in cases of denture-induced candidosis. Sodium hypochlorite has a fungicidal effect at 0.02 % concentration (Webb *et al.*, 2005). Moreover, disinfection of soft lining materials in dilute sodium hypochlorite is more effective than exposure to microwave energy (Baysan *et al.*, 1998). However, the limitation of using this cleanser is its corrosive effect on metals (Budtz-Jorgensen, 1979), and it may deteriorate soft lining materials if used incorrectly (Nikawa *et al.*, 2003). Although chemical disinfection has been largely recommended, it has been demonstrated that the hardness, strength and colour stability of denture base materials can be significantly affected by disinfectant solutions (Asad *et al.*, 1992, 1993).

Immersion of dentures in 0.2 % chlorhexidine has shown an anti-inflammatory effect in patients with denture-induced candidosis (Olsen, 1975) and the emergence of resistance to chlorhexidine has not been reported clinically (Thurmond *et al.*, 1991). Chlorhexidine and nystatin are also effective in treating candidal inflammation when used as denture disinfectants (Nualwan, 1976). Other agents have also been used, for example, combining coconut soap (a natural antibacterial soap mainly used for cleaning and hand washing) and 0.5 % sodium hypochlorite has significantly reduced denture-induced candidosis (Barnabe *et al.*, 2004).
1.6.2.2 Modification of surface properties

Altering the chemical composition of the denture fitting surface may reduce microbial adhesion and biofilm formation (Dhir et al., 2007). Modifying poly (ethyl urethane) surfaces by adding 6 % polyethylene oxide results in inhibition of biofilm formation and prevention of C. albicans adhesion (Chandra et al., 2005). In addition different surface finishes affect the adherence of C. albicans, with greater adherence to the rougher surface (Radford et al., 1998; Frade and Arthington-Skaggs, 2011; Li et al., 2012). Moreover, glazing the fitting surface of the denture would reduce the microbial count versus the untreated surface (Monsenego, 2000).

Enamel pellicle contains histatins (antimicrobial peptide), IgA, amylase, mucin and stathrins. In contrast, statherins and histatin are absent in denture pellicle which is partly responsible for the lack of anionic charge in denture bases, and as a result enhances the adhesion of C. albicans to these surfaces (Edgerton and Levine, 1992). In addition, modification of denture base resins by increasing the amount of methacrylic acid in place of methyl methacrylate monomer during processing results in a decrease in adhesion of C. albicans (Park et al., 2003). Adding phosphate-containing monomer (anionic charge) to denture bases also inhibits adhesion of C. albicans and evaluation of the physical properties of a modified denture resin at 10 % has been found to be within the American Dental Association (ADA) specifications and appropriate for denture fabrication (Dhir et al., 2007).

1.6.2.3 Prosthetic treatment

Fabrication of new well-fitting dentures has been suggested as a solution for denture-induced candidosis on the basis that trauma is an important aetiological factor in this condition (Nyquist, 1952; Emami et al., 2008). Removing trauma,
by correcting faulty occlusion and providing balanced occlusion, has been demonstrated to resolve localised simple denture-induced stomatitis lesions (type I, § 1.3.2) but *Candida* induced lesions (type II & type III, § 1.3.2) are not resolved by prosthetic treatment (Budtz-Jorgensen and Bertram, 1970b). In contrast, Richie *et al* (1969) reported a positive effect of prosthetic treatment whatever the cause of the inflammation (trauma or *Candida*). Healing following using relining with tissue conditioners was reported, possibly because it distributes the load more evenly and reduces the trauma (Budtz-Jorgensen and Bertram, 1970b). However, denture lining materials needs to be changed regularly (Budtz-Jorgensen and Bertram, 1970b), because it enhances candidal growth more than acrylic surfaces (Bulada *et al*., 2004). Furthermore, providing lower implant overdentures may reduce oral mucosal trauma and control denture-induced candidosis, because more stability and consistent biting force vectors can be offered by implant retained denture (Emami *et al*., 2008).

**1.6.2.4 Microwave energy as a disinfectant**

*C. albicans* can penetrate into the fitting surface of acrylic denture resin and between its polymeric chains (Levin, 1973), resulting in difficulty in eradication of the yeast by mechanical (Chan *et al*., 1991) as well as chemical cleaning methods (Keng and Lim, 1996). The use of microwave energy to disinfect dentures has been suggested to overcome the drawbacks of chemical disinfection (Dixon *et al*., 1999), as chemical disinfection can affect hardness, flexural strength and colour stability (Asad *et al*., 1992; Neppelenbroek *et al*., 2005) as well as promoting tarnish and corrosion of denture metal components and bleaching of acrylic resin (Neill, 1968; Shen *et al*., 1989). It has been proven that microwave denture disinfection is effective in treating
denture-induced candidosis (Webb et al., 2005). The thermal effect of microwave exposure is thought to cause destruction of microorganisms and microwave irradiation promotes structural alteration in the cell wall of *C. albicans* (Campanha et al., 2007). While microwave exposure of 10 min (604 W) results in dimensional changes to denture, 6 min exposure (331 W) was found to be sufficient for disinfection while maintaining dimensional stability (Thomas and Webb, 1995). The superiority of microwave disinfection over chemical disinfection has been demonstrated (Webb et al., 1998a; Banting and Hill, 2001) and it is considered as an effective and quick alternative to sodium hypochlorite because of the clinical disadvantages of the latter. However, microwave energy disinfection does not overcome the problem of metal components of the denture when using a conventional microwave oven (Baysan et al., 1998) and disinfection of soft lining material by soaking in sodium hypochlorite proved to be more effective than exposure to microwave energy (Rohrer and Bulard, 1985; Baysan et al., 1998).

### 1.6.2.5 Diode laser irradiation

The ability of low-power lasers to destroy fungi and bacteria has been reported (Wilson and Mia, 1993). These results indicate that low-power lasers may be a promising treatment modality for denture-induced candidosis (Maver-Biscanin et al., 2004). This treatment may eradicate or reduce the fungal load on the fitting surface of the denture and as a result reduces the recurrence rate of this condition (Wilson and Mia, 1993).
1.6.3 Treatment modalities directed toward both oral mucosa and denture base

1.6.3.1 Intra-oral delivery of therapeutic agents

Biomaterial chemical alteration, such as antimicrobial impregnation into acrylic, aims to improve the fungal control and to resist biofilm formation (Cuéllar-Cruz et al., 2012). The feasibility of using drug delivery systems by incorporation of antifungal or antimicrobial agents in denture acrylic resin or soft liners has been investigated (Quinn, 1985; Schneid, 1992; Truhlar et al., 1994; Patel et al., 2001; Amin et al., 2009; Ryalat et al., 2011) (Figure 1.6). The use of polymerised acrylic as carriers for drugs orally (Douglas, 1977; Amin et al., 2009) is a method to extend the duration of effective therapy. Similarly, soft liners have been used as carriers for antifungal drugs in treating denture-induced candidosis (Douglas and Walker, 1973; Schneid, 1992; Chow et al., 1999).

Figure 1.6 Schematic diagram showing the principle of controlled-release drug delivery system
The idea of using controlled-release drug systems is to release the drug at a specified rate from a protected reservoir and to maintain an ideal therapeutic level over the required time period for treatment (Brook and van Noort, 1984). Many advantages have been reported for controlled release systems; they increase patient convenience and reduce the need for rigid compliance with frequent dosing regimes. Direct delivery of a drug to the site of infection also reduces the systemic effect. Further, the peaks and troughs of conventional therapy are eliminated, and replaced by continuous delivery and availability of the drug, thus the resultant risks of sub-therapeutic or toxic drug levels are also reduced, while the constant level results in improved therapeutic outcome (Brook and van Noort, 1984) (Figure 1.7).

Figure 1.7 The difference in the achieved drug levels between the conventional treatments and the controlled drug delivery systems
Different combinations of polymers impregnated with antifungal and/or antimicrobial agents have been studied. Antifungal agents, impregnated into a tissue conditioner, result in inhibition of candidal growth, demonstrating the release of the drugs from the liner (Schneid, 1992). Ammonium quaternary compounds can be incorporated in the resin powder and polymerised with acrylic denture base. The resultant polymeric system has inherent antiseptic properties both fungistatic and bactericidal and seems a promising management of denture-induced candidosis (Pesci-Bardon et al., 2004).

Owing to the proven antifungal efficacy of chlorhexidine there has been considerable interest in the application of this agent, through carriers and slow-release polymeric systems (Amin et al., 2009; Ryalat et al., 2011). The feasibility of establishing intra-oral controlled-release delivery system for chlorhexidine was investigated using different carriers (Lamb and Martin, 1983; Brook et al., 1986). Varnishes (Balanyk and Sandham, 1985), and ethyl cellulose films (Friedman and Golomb, 1982; Hirshfeld et al., 1984) have been investigated to be used as delivery systems for chlorhexidine. Cold and heat cured acrylics containing chlorhexidine have been used as carriers and assessed for their potential to release chlorhexidine (Amin et al., 2009; Ryalat et al., 2011). The release of chlorhexidine out of impregnated polymeric delivery systems can be detected for more than 100 days and the polymerisation of the acrylic does not seem to affect the efficacy of chlorhexidine (Addy, 1981). However, the physical properties of the impregnated resin are affected due to the presence of chlorhexidine particles, which may dissolve and result in porosity in the acrylic base (Addy and Handley, 1981). This effect can be neglected by employing
these materials as temporary measures (e.g. relining or rebasing materials) in existing prostheses.

Delivering chlorhexidine over many weeks using these systems reduces patient involvement in drug administration and increases patient satisfaction and prevents the local side effects of chlorhexidine because of the targeted delivery (Friedman and Golomb, 1982). These formulations have fewer side effects compared with the conventional forms, due to the continual presence of the drug at the site of infection, and less amount of drug is needed to achieve the therapeutic effect (Brook and van Noort, 1984). In addition, an intra-oral delivery system for chlorhexidine could be with high benefit for physically or mentally compromised patients (Mirth et al., 1989). Sustained topical release of chlorhexidine has been reported by Riggs et al. (2000), who used a self-curing system based on poly (ethyl methacrylate) and tetrahydrofurfuryl methacrylate polymeric system (PEM/THFM).

Many factors should be considered to design a controlled-release drug delivery system for intra-oral use (Brook and van Noort, 1984):

- Polymers used to produce the delivery devices should be biocompatible and safe.
- The acrylic delivery device should be well tolerated by the patient, inexpensive, and easy to use in the mouth.
- Devices must be stable in oral environment with very low solubility.
- Adjustment of release rate in the device should be possible by minor alteration in the design.
Monolithic delivery device (impregnate the drug with the polymer) is favoured over a reservoir delivery device (an inert diffusion barrier surrounding solid core of drug).

Release rates must be constant and predictable; the release should be consistent with the clinical needs and within the recommended ranges.

To improve drug release, high water uptake of the delivery system permits drug to leach out of the material.

PEM/THFM polymeric system has a superior drug release characteristic compared with methyl methacrylate and n-butyl methacrylate based systems (Patel et al., 1994). This system is ductile in nature, which is a good characteristic for prostheses (Patel and Braden, 1991c). Moreover, the same system has been reported to enjoy excellent biological properties (Pearson et al., 1986; Wyre and Downes, 2000). In addition, the suitability of PEM/THFM as a delivery system seems to be related to the high water uptake of this acrylic system: this would account for enhanced release of the medicament (Riggs et al., 2000).

Some of the previously discussed studies that investigated the possibility of introducing a system for intra-oral delivery of therapeutic agents employed chlorhexidine for the treatment of denture-induced candidosis. Chlorhexidine impregnated acrylics of a suggested polymeric delivery system were soaked in distilled water and the eluates were tested for the presence of chlorhexidine. The methods of detecting the released drug varied, both in type and sensitivity, among investigators. Addy (1981) used spectrophotometric and agar diffusion measurements to detect chlorhexidine leaching; Riggs et al. (2000) employed a proton nuclear magnetic resonance spectroscopy (NMR) for the same purpose, whereas
Patel et al (2001) used ultraviolet spectrometry in their measurements to detect chlorhexidine release. Ryalat et al (2011) demonstrated that chlorhexidine could be released steadily from self-cured poly (methyl methacrylate) acrylic throughout the test period using high performance liquid chromatography (HPLC).

Few studies investigated whether the concentration of the released chlorhexidine into the surrounding medium was effective microbiologically. Patel et al (2001) used a turbidimetric measurement of candidal growth after exposure to an experimental drug release device both with and without chlorhexidine. It was found that the chlorhexidine-containing test samples inhibited candidal growth in vitro. The number of surviving Colony Forming Unit/ml (CFU/ml) of culture medium was substantially reduced for the test sample compared to controls. Another study used a well diffusion test to check the antifungal efficiency of the leachates, where chlorhexidine leachates have shown larger inhibition zones compared to fluconazole (Amin et al., 2009).

Different methods can be applied to assess the presence and the amount of the released material under study. Examination using the UV spectra (Ultra-Violet spectrophotometer) of the solution is one method that can be used (Patel et al., 2001). Another universal type of analytical procedures that can be applied with high efficiency is HPLC. It is used for a wide range of applications and offers significant advantages in the analysis of pharmaceutical formulations, biological fluids, synthetic and natural polymers, a variety of inorganic substances and trace element contaminants (Braithwaite and Smith, 1996). However, HPLC is technically demanding (Amin et al., 2009; Andes et al., 2009), whereas spectrophotometry is easy to apply and provides reliable results of drug concentrations in solutions (Wilson and Wilson, 1993).
Bioassay is a microbiological assay used to measure the concentration or potency of antimicrobial agents against a suitable microbe under standard conditions by measuring the area of the zone of inhibition by a test substance and comparing with that of standards with known concentrations (Zuluaga et al., 2009). Such a method has particular advantages, it is quantitative and qualitative method as it estimates both the concentration and the potency, accurate, rapid and cost-effective (Andes et al., 2009).
1.7 Spectrophotometer

1.7.1 Introduction

Spectrometry is a technique used to measure the concentration or amount of a given material. The instrument that performs these measurements is a spectrophotometer or spectrograph (James, 2007). A spectrometer (spectrograph or spectroscope) is an optical instrument used to measure properties of light over a specific portion of the electromagnetic spectrum typically used in spectroscopic analysis. The measured variable is most often the intensity of the light. The independent variable is usually the light wavelength, normally expressed in nanometers. A spectrometer is used in spectroscopy for producing spectral lines and measuring their wavelengths and intensities (Ingle and Crouch, 1988). Spectrometers operate over a very wide range of wavelengths, from gamma rays and X-rays into the far infrared (IR) (Skoog et al., 1997). Figure 1.8 demonstrates the electromagnetic spectrum.

![Figure 1.8 Electromagnetic spectrum](image)

Figure 1.8 Electromagnetic spectrum
1.7.2 The principle of spectroscopic process

A light source (usually either a UV deuterium lamp or a VIS tungsten or tungsten/halogen lamp) is used to create two identical beams of light. A prism or diffraction grating is used to separate the beam of light from a visible (VIS) and/or UV light source into its component wavelengths (James, 2007). This light passes through a monochromator to select a single wavelength. One of these beams passes through the sample itself and the other beam passes through the reference cell, which usually contains everything that is in the sample to be measured except for the molecules under investigation (James, 2007).

To summarise, UV and visible spectrometers measure the amount of ultraviolet and visible light transmitted or absorbed by a sample placed in the spectrometer. The wavelength at which a chemical absorbs light is a function of its electronic structure and the intensity of the light absorption is related to the amount of the chemical between the light source and the detector (concentration), so a UV/VIS spectrum can be used to identify different chemical species (Ingle and Crouch, 1988) (Figure 1.9).

**Figure 1.9 Typical UV-Vis spectrophotometer**
1.7.3 Instrumentation

Most modern UV spectrometers consist of the following (Skoog et al., 1997; James, 2007) (Figure 1.10):

- **Light sources**: two sources are required to illuminate the entire UV-VIS band, the lamps that illuminate the entire band of UV or VIS light are:
  - Deuterium lamp – covers the UV (200-330 nm)
  - Tungsten lamp – covers the VIS (330-700 nm)
- **Monochromator (grating or prism)**: this gradually changes the small bands of radiation: these radiations to be sent to beam splitter.
- **Beam splitter**: this sends a separate band to a cell container.
- **Cuvette**: is a transparent container, containing the sample and the reference solution. It can be made of plastic, glass or quartz. Only quartz is transparent in the full 200-700 nm range; plastic and glass are only suitable for visible spectra.
- **Detector**: this measures the difference between the transmitted light through the sample versus the reference light and sends this information to the recorder which can be a computer and/or a printer.

![Figure 1.10 Basic components of spectrophotometer](image)
1.7.4 Calculations and analysis

The reference beam in the spectrometer travels from the light source to the detector without interacting with the sample. The sample beam interacts with the sample exposing it to ultraviolet light of continuously changing wavelength. When the emitted wavelength corresponds to the energy level which promotes an electron to a higher molecular orbital, energy is absorbed. The detector records the ratio between reference and sample beam intensities ($I_0/I$) (Ingle and Crouch, 1988). At the wavelength where the sample absorbs a large amount of light, the detector receives a very weak sample beam. The intensities of these light beams are then calculated by electronic detectors and compared. The intensity of the reference beam, which should have suffered little or no light absorption, is defined as $I_0$. The intensity of the sample beam is defined as $I$ (Skoog et al., 1997). The ratio of reference beam and sample beam intensities sent to computer (Figure 1.11).

![Diagram of spectrometer setup](image)

**Figure 1.11  Sample and reference beam intensities**
The transmittance (T) is defined as the ratio of the intensity of light that gets through the sample cell over the intensity of light that goes through the reference cell (Ingle and Crouch, 1988).

\[
T = \frac{I_{\text{sample}}}{I_{\text{reference}}}
\]

The absorbance (A) or Optical Density is defined as

\[
A = \log \left( \frac{I_0}{I} \right)
\]

The absorbance of a solution increases as attenuation of the beam increases, thus absorbance is directly proportional to the path length, \( l \), and the concentration, \( c \), of the absorbing sample. The Beer-Lambert law gives the relationship between concentration, absorbance and transmitted light intensity:

\[
A = \varepsilon l c
\]

Where \( \varepsilon \) is called the extinction coefficient, \( l \) is the path length of the sample cell, and \( c \) is the concentration.

1.7.5 The spectrum

The typical layout of the spectrum output is demonstrated in Figure 1.12, it consists of two axes (James, 2007). The x-axis of the spectrum is in wavelength; 200-330 nm for UV, 200-700 nm for UV-VIS determinations, the peak maxima are simply reported as a numerical list of “lamba max” values or \( l_{\text{max}} \) and the y-axis of the spectrum is in absorbance, \( A \) (Ingle and Crouch, 1988).
1.7.6 Practical application of UV spectrophotometry

UV spectrophotometry is a well known precise analytical quantitative instrument (James, 2007). It has been used for many applications (Wilson and Wilson, 1993; Patel et al., 2001). Using the absorbance curves of known concentrations the concentration of unknown materials can be predicted (Patel et al., 2001). In addition, it can be used to evaluate the proper irradiation wavelengths for photochemical experiments (via $\lambda_{\text{max}}$ and molar absorptivity), or the design of UV resistant paints and coatings (Skoog et al., 1997). It is most useful in combination with NMR and IR data to reveal unique electronic features that may be ambiguous in those methods (Ingle and Crouch, 1988). The most common use of UV spectrophotometry is as a detection device for HPLC; since UV spectrophotometry is used for solution phase
samples versus a reference solvent this is easily incorporated into liquid chromatography (LC) design (Skoog et al., 1997). Although UV spectrophotometry was the first organic spectral method to be used, it is rarely used as a primary method for structure determination (Ingle and Crouch, 1988).
1.8 Properties of PEM/THFM acrylic system

Denture liners are widely used to relief pain and to improve the fit between the denture surface and the underlying mucosa (Mack, 1989; Anil et al., 1999). In addition, these liners can be used as drug carriers by being impregnated with antifungal agents (Schneid, 1992; Truhlar et al., 1994; Chow et al., 1999). Denture liners can be provisional or permanent, acrylic or silicone based, and can be chemically or heat cured (Ergun and Nagas, 2007). Provisional liners are those lining materials that are designed for intra-oral use for up to 30 days (Ergun and Nagas, 2007). Acrylic based lining materials are basically composed of a polymer powder and a liquid of methacrylate monomer (Ergun and Nagas, 2007). PEM/THFM is an autopolymerising acrylic resin which has been suggested as a drug carrier in vitro with promising results (Patel et al., 2001). Although this system has been suggested to be used for a short-term clinical service, it should fulfil certain standards to optimise its clinical performance (Patel et al., 2001).

Ideally, a denture liner should fulfil certain criteria to function effectively within the required frame time. They should satisfy several physical, mechanical, biological and aesthetic requirements. However, materials deteriorate with aging after being exposed to environmental conditions and incorporation of antifungals into liners can reduce the mechanical and physical properties of the liner and affect its longevity (Addy and Handley, 1981; Faber et al., 2005). As a result it is highly important to test the physico-mechanical properties of these impregnated liners to assess clinical performance and serviceability (Aydin et al., 1999).
1.8.1 Testing procedures of serviceability of acrylic liners

The functionality of prosthetic materials can be tested via their mechanical properties under different simulated conditioning environments (Mese and Guzel, 2008). Such mechanical testing determines the appropriateness of the tested material for clinical service. Moreover, physical properties such as water absorption and colour stability are important properties to evaluate the longevity of dental liners (Anil et al., 1999; Wei et al., 2011). Furthermore, assessment of prosthetic material compatibility or toxicity by considering the residual monomer content in the acrylic is crucial to prove its clinical suitability (Vallittu et al., 1995; Bural et al., 2011).

Important clinical properties to be investigated to determine the durability of denture liners (such as PEM/THFM) for short-term use are thoroughly described in the next section.

1.8.1.1 Shear bond strength

Denture liner materials can only be successful if a satisfactory bond between the liner and the denture base can be achieved (Aydin et al., 1999; Mese and Guzel, 2008). A durable bond is highly important to optimise the clinical performance of the liners for the desired service time (Maeda et al., 2012). Bond failure between the liner and the denture is a serious problem that renders the liner useless (Aydin et al., 1999). This failure could be due to structural differences between the two different materials (Aydin et al., 1999).

The durability of the bond between the liner and the denture base is the most important factor for successful use, as the bond should be serviceable for the desired treatment time period (Aydin et al., 1999). Various tests have been employed to
assess the bond strength of denture liners; Maeda et al (2012) evaluated bond strength of eight denture liners using a peeling test; Kulak-Ozkan et al (2003) applied a tensile test to measure the bond strength of 6 denture materials; another study has investigated the bond strength using shear bond test (Hatamleh and Watts, 2010).

All the above tests have been criticised. The peel test, despite its similarity to the clinical forces, is highly influenced by compliance and thickness of materials; and bond failures being mainly cohesive within peeled materials (Omer, 1994; McCabe et al., 2002). The shear bond test is argued to concentrate stresses at the margins, and it is affected by the selected deformation rate and the specimen thickness (Al-Athel and Jagger, 1996). The tensile test is an inherently sensitive method, and reflects the tensile strength of the material itself rather than its bonding to the substrate (Stamatacos-Mercer and Hottel, 2005). Furthermore, tensile bond strength values reported for identical materials varied significantly depending on the condition and surface area of specimens (Stamatacos-Mercer and Hottel, 2005). Accordingly, there is no single test that can represent all acting forces in clinical situations and all have their inherent disadvantages. However, the shear bond test can be related to the loads that affect the lining materials during function and can be clinically relevant (Hatamleh and Watts, 2010).
The shear bond (SBS) can be calculated according to the following equation:

\[
\text{Eq. (1.4)}
\]

Where \( F \) stands for maximum force to failure expressed in (N), \( A \) is the area of attachment expressed in (mm\(^2\)); SBS represents the shear bond strength (MPa).

Other mechanical properties e.g. flexural strength and Young’s modulus have been studied for PEM/THFM polymeric systems and are favourable for use as a temporary material (Patel and Braden, 1991c).

1.8.1.2 Water absorption

Although water absorption can dramatically affect dimensional stability, bond strength and colour stability, its importance is often overlooked (Ergun and Nagas, 2007; Mese and Guzel, 2008). When the liner absorbs water, it swells and stresses will build up at the bond interface (Aydin et al., 1999). Moreover, water absorption enhances the elution of leachable particles within the matrix (Wei et al., 2011). However, this can be a benefit in drug carrier liners to enhance drug release (Riggs et al., 2000).

Water absorption can be monitored by placing acrylic liner specimens of known dimensions into distilled water at constant temperature. The percentage absorption can be calculated as a function of time (Riggs et al., 1999) using the following measurements:

\( m_1 \): The mass at the baseline, \( m_2 \): recorded mass at any given time, \( m_3 \): The mass at equilibrium. The percentage of mass change (\( M_g \)) can be calculated by:
Few studies have evaluated the water absorption characteristics of PEM/THFM polymeric systems per se. Riggs et al (1999) investigated the behaviour of PEM/THFM in water and found that the water uptake is high: up to 70 % over 3 years. Additionally, higher water absorption was reported by Riggs et al (2000) after impregnation of PEM/THFM with chlorhexidine. This unique water absorption capability is a critical attribute for this system to be used as a delivery system with enhanced release (Riggs et al., 2000).

1.8.1.3 Degree of conversion

Ideally, the polymerisation reaction of acrylic resins should result in conversion of all monomer into polymer (Vallittu et al., 1995). In practice some of the monomer does not convert resulting in residual monomer which might leach into human saliva (Baker et al., 1988). The level of residual monomer is related to the degree of conversion (DC) which can be achieved using chemical activator in autopolymerising acrylic resin (Vallittu et al., 1995; Vallittu, 1996). DC is a critical parameter that influences the biocompatibility and the mechanical and physical properties of dental polymers (Baker et al., 1988; Karmaker et al., 1997; Boeckler et al., 2008) and assessing the DC is a valuable mean to evaluate the mechanical properties of a polymer and its clinical performance (Stansbury and Dickens, 2001).

DC can be determined by measuring the percentage of aliphatic bonds (C=C) that have been converted into single bonds to form an extended network.
polymer (Stansbury and Dickens, 2001). The extent of polymerisation can be affected by many factors: powder-to-liquid ratio, mixing method and resin composition (Duray et al., 1997). Moreover, it has been shown that adding chlorhexidine to PEM/THFM hinders the polymerisation and higher levels of residual monomer are leached compared to the parent polymer (Riggs et al., 2000).

Few studies have been carried out to assess biocompatibility of PEM/THFM systems. Riggs et al. (2000) showed that residual monomer was detected in leachates from PEM/THFM acrylic polymer. Pearson et al (1986) have shown that this acrylic system is non-irritant and well tolerated by dental pulp tissue and it has a potential to support bone and cartilage repair (Wyre and Downes, 2000).

Fourier transform infrared (FTIR) spectrometer is a widely used non-destructive molecular spectroscopic method (Duray et al., 1997). It measures the interaction of a material with the emitted infrared beam (Celina et al., 1997) and has proved to be a robust instrument for identification and monitoring setting reactions of a wide range of different chemical compounds. Many advantages have been reported; it is a universal technique which can be used for any material, a wide range of information can be obtained, and it is fast, inexpensive and easy to use (Guerra et al., 1996; Stansbury and Dickens, 2001).

1.8.1.4 Colour stability

Light represents a narrow band in the electromagnetic spectrum (400-700 nm) which is the only detectable range by human eye (Joiner, 2004). Within this visible range the shortest wavelength (400 nm) corresponds to blue/violet colour and the longest wavelength (700 nm) corresponds to deep red. Perception of colour is related to the
physical interaction of light energy with the object to be viewed and the individual observer, however, colour perception is subjective and varies according to age, mood of observer and the range of wavelengths of light source (Joiner, 2004). Consequently, universal colour quantifying methods are highly needed.

A number of colour scales have been evolved in attempt to communicate colours universally (Sproull, 1973). The Munsell colour system describes colours in three dimensions: hue (colour family), chroma (colour saturation) and value (lightness) (Figure 1.13) (Joiner, 2004).

![Munsell Colour System](https://example.com/munsell.png)

**Figure 1.13 Munsell Colour System (Nimeroff and Judd, 1968)**

Hue is the ordinary colour name, and is divided into ten categories according to the Munsell colour system; five main hues (Red, Yellow, Green, Blue, and Purple) and five intermediate hues. Chroma defines the purity of a colour, with lower chroma colours
being less pure (more washed out). Saturated colours such as red, yellow, green, blue, and purple are highly chromatic colours (in their highest purity). However, non-saturated colours are the ones that are mixed with white, black or grey. In a scale of saturation from 0 to 100 %, for instance, achromatic colours (black, white, greys) have 0 % saturation; while the main spectral colours have 100 % saturation (they are the purest visible colours). Value (Lightness) indicates whether the colour appears to emit more or less light and may also be referred to the brightness of the colour and it ranges from black to white (Nimeroff and Judd, 1968).

Other colour identification systems have been developed based on these distinct colour variables such as the tristimulus system and CIE L*a*b* colour system which are the most popular colourimetric systems (O’Brien et al., 1997).

**The tristimulus colour system**

The tristimulus system is based on the three primary colours, red, green and blue against which visually perceived colours are mapped. The three variables are expressed as X, Y, and Z, respectively, and are called tristimulus values. These values specify the colour and the visually perceived reflectance, and are represented so that the Y value equals the sample's reflectivity when visually compared to a standard white surface by a standard (average) viewer under average daylight. The tristimulus values can also be used to determine the dominant spectral wavelength (which is related to the hue) of a given sample. A standard chromacity diagram has been approved by the Commission Internationale d’Éclairage (CIE) and it is basically based on x, y, and z values (Figure 1.14). X, y, and z values can be calculated as follows:

\[ x = X/(X + Y + Z), \ y = Y/(X + Y + Z), \ \text{and} \ z = Z/(X + Y + Z) \]  

(Nimeroff and Judd, 1968).
The CIE $L^* a^* b^*$ Colour System

This system describes three colour values of $L^*$, $a^*$ and $b^*$, to facilitate classifying colour differences numerically. It describes 3-dimensions of the colour ($L^*a^*b^*$) in the colour space (Figure 1.15). The $L^*$ parameter reflects the degree of lightness and darkness which ranges from black (0) to white (100), whereas $a^*$ and $b^*$ are chromacity which indicate red or green chroma ($+a^* = red$, $-a^* = green$) and yellow or blue chroma ($+b^* = yellow$, $-b^* = blue$). The colour changes, $\Delta E^*$, were calculated from the three colour values $L^*$, $a^*$ and $b^*$ according to the following formula:

$$ - \quad \text{Eq...(1.6)} $$
Where:

\((L^*, a^*, b^*)_1\) the values before aging
\((L^*, a^*, b^*)_2\) the values after aging

**Figure 1.15 The CIE L*a*b* Colour System**

The major advantage of the CIE L*a*b* system is that the colour differences can be expressed numerically and can be transmitted into visual perception and clinical significance. For colour analysis, the total reflected light at each wavelength in the visible spectrum (400-700 nm) is measured and recorded. The total amount of reflected light, dominant wavelength (actual colour) and excitation purity (chroma) are calculated in accordance with the CIE chromatography diagram, using a standard white light source defined as the spectral function of a 100 W incandescent lamp at a colour temperature of 2800 Kelvin (Nimeroff and Judd, 1968).
1.9 Summary

Denture-induced candidosis is the most frequent clinical presentation of oral candidal infections, characterised by an inflamed mucosa under upper dentures. It is a multifactorial condition and commonly affecting elderly denture wearers and especially a serious problem in immunocompromised patients. *C. albicans* is the main species associated with oral candidosis. However, other *Candida* species have been isolated, such as *C. glabrata*, which may play an important role in the pathogenesis of oral candidosis. The presence of non-*albicans Candida* species is a main concern because they are often resistant to conventional antifungal treatment. Topical and systemic antifungal treatment options are available including the classic polyenes,azole antimycotics and the promising recently used echinocandins. Despite the availability of a wide range of treatment options, therapeutic failures are commonly documented.

Biofilm is the most common form of growth of yeasts in nature. Of all human microbial infections, over 65 % relate to biofilm. Moreover, 80 % of microorganisms live in organised complex structured communities (biofilm) (Costerton et al., 1999). Oral candidosis is a mixed biofilm infection which provides further challenges for its management (Ramage et al., 2005). Biofilm lifestyle is commonly associated with poor drug penetration and antimicrobial recalcitrance as well as resistance for host defence mechanisms (Cowen et al., 2002; Ramage et al., 2005).

Fluconazole is commonly used to treat denture-induced candidosis as it has a broad antifungal activity. It is well tolerated with few side effects; however, emergence of resistance is the main clinical concern as well as the reduced efficiency against
non-albicans Candida species such as *C. glabrata* and candidal biofilms (Rautemaa and Ramage, 2011). Interestingly, topical alternative therapeutic agents such as chlorhexidine have proven exceptional efficiency against *Candida* species (Redding *et al.*, 2009; Rautemaa and Ramage, 2011). In addition, chlorhexidine has unique anti-biofilm activity with low MIC values (Lamfon *et al.*, 2004; Redding *et al.*, 2009).

One of the main issues in topical oral treatment is the washing effect of the saliva and the musculature which reduce the availability of the drugs to sub-therapeutic levels. Moreover, a rigid patient compliance for frequent drug administration is paramount to achieve optimal outcome. A local delivery system is an attractive option to maintain therapeutic drug levels at the site of pathology. It is more convenient for the patient as it is a self-release system. Delivery systems have been studied extensively using different polymers impregnated with different therapeutic agents and promising results have been reported.

A self-cured PEM/THFM polymeric system is a promising delivery system. Its biocompatibility, water uptake properties and the negligible impact on the activity of the impregnated drugs are the main attributes characterising this polymeric system.

It may be possible to add a new therapeutic modality to the available antifungal treatment options by establishing an antimicrobial delivery device. The overall aim of this research is to identify the potential efficiency of this therapeutic option and to accurately identify its suitability and durability in an oral simulating environment over the required treatment period. A PEM/THFM polymeric system impregnated with chlorhexidine or fluconazole is to be investigated. Figures 1.16 and 1.17 show
the 2-D and 3-D chemical structures of tetrahydrofurfuryl methacrylate (THFM), poly ethyl methacrylate (PEM), fluconazole and chlorhexidine.

In order to establish an effective antimicrobial delivery device it is necessary to:

- Release the impregnated antimicrobials in adequate therapeutic concentrations during the intended treatment duration.
- The released concentrations should be microbiologically effective against wide range of commonly associated *Candida* species.
- The antimicrobial delivery device should be effective against both the planktonic and the biofilm candidal lifestyle.
- The impact of the drug impregnation on the mechanical and physical properties of the delivery polymeric system should be minimal and within acceptable ranges.
Figure 1.16  2-D and 3-D chemical structure of THFM (a), PEM (b) (produced using Advanced Chemistry Development simulator software, version 11.01)
Figure 1.17 2-D and 3-D chemical structure of fluconazole (a), chlorhexidine (b) (produced using Advanced Chemistry Development simulator software, version 11.01)
CHAPTER 2

STATEMENT OF THE PROBLEM,

AIMS & OBJECTIVES & WORK PLAN
2.1 Statement of the problem

Denture-induced candidosis is a common disease in elderly denture wearers with a prevalence of 45-70% (Figueiral et al., 2007; Dagistan et al., 2009). It is a multifactorial disease and *Candida albicans* is the principal causative agent. Other *Candida* species such as *C. glabrata* are commonly identified especially in medically compromised patients (Coco et al., 2008; Rautemaa and Ramage, 2011). Many therapeutic modalities are available ranging from denture disinfection to systemic antifungal therapy (Webb et al., 1998c; Uludamar et al., 2011). Despite this the recurrence rate of denture-induced candidosis is high (Lal et al., 1992; Cross et al., 2004). It has been suggested that this is due to poor access of the antifungals onto the fitting surface, their poor penetration into the microbial biofilm on the porous denture material, as well as their rapid clearance by saliva and tongue movements (Addy and Fugit, 1989; Cross et al., 2004; Ramage et al., 2005; Seneviratne et al., 2008).

Being the “disease of the diseased”, oral candidosis predominantly affects immunosuppressed and medically compromised patients (Coco et al., 2008). In these high-risk patients, the oral cavity may provide a source of systemic candidal infection (Rautemaa and Ramage, 2011). Oral candidosis has become a significant challenge in patients with persisting risk factors and a recurrent need for antifungal treatment (Rautemaa and Ramage, 2011). In particular, repeated courses of fluconazole have been shown to form a risk for persistent colonisation with microbiologically and clinically resistant *Candida* (Johnson et al., 1995). Oral candidosis is a mixed biofilm infection which provides further challenges for its management.
(Ramage et al., 2005). Biofilm lifestyle is commonly associated with poor drug penetration and antimicrobial resistance (Cowen et al., 2002; Ramage et al., 2005).

A number of antifungal agents can be used for the management of fungal infections (Samaranayake et al., 2009). However, the choice of antifungals suitable for the treatment of oral candidosis is limited (Rautemaa and Ramage, 2011). Fluconazole is a widely used systemic antifungal agent which is well tolerated with low toxicity and mild side effects (Samaranayake et al., 2009). However, in elderly patients with reduced saliva production, there is a risk for inappropriately low therapeutic drug levels in the oral cavity and emergence of resistance (Siikala et al., 2010). Moreover, non-albicans Candida species such as C. glabrata and C. krusei intrinsically resistant to fluconazole are common causes of oral candidosis (Heimdahl and Nord, 1990; Bagg et al., 2003; Coco et al., 2008). In addition, its penetration into candidal biofilm is poor leading to low drug concentrations which is a potential risk of selection and development of resistant strains and it does not offer any cure to the Candida infested the fitting surface of the denture (Ramage et al., 2002a).

Nystatin is a highly effective topical antifungal with few drug interactions; however, its four-times daily dosage is a significant challenge for patient compliance (Blomgren et al., 1998; Su et al., 2008). Echinocandins are highly effective agents against Candida and Candida biofilms (Ramage et al., 2002b); however, its availability only as intravenous formulations and its high cost negate its use for treatment of oral candidosis (Rautemaa and Ramage, 2011).

The persistent fungal presence on the fitting surface of dentures often leads to cross infection and recurrence of the mucosal lesions. In addition, the number of medically
compromised patients, who are in a high risk of infection and limited treatment options, is increasing. Moreover, denture-induced candidosis is a mixed biofilm infection which provides multiple challenges for its management. Based on these facts, developing new biomaterials with antimicrobial properties is of unquestionable importance.
2.2 Aims and Objectives

The aims of the present research were to establish whether a polymeric delivery device based on auto-polymerising denture base lining polymer, poly (ethyl methacrylate) and tetrahydrofurfuryl methacrylate (PEM/THFM), for sustained delivery of antifungal agents (chlorhexidine and fluconazole), could be developed for the use in the treatment of denture-induced candidosis. Additionally, the serviceability of the lining under study has been examined (through in vitro studies).

The specific objectives were to:

- investigate the antifungal activity of chlorhexidine against a large number of *Candida* species, and compare its activity against fluconazole.
- evaluate the efficiency of the PEM/THFM denture liner to release the impregnated antifungal agents (quantitative assessment).
- verify the antifungal activity of the released antifungal agents (qualitative assessment) against planktonic and biofilm lifestyles.
- assess a range of clinically important physical and mechanical properties for the impregnated liner. Those properties are shear bond strength, water absorption, degree of conversion and colour stability.

This thesis is structured to contain an overall Introduction, Aims and Objectives and Methods sections followed by seven chapters with specific themes and an overall Discussion and Summary.

A flow chart showing the work plan is presented diagrammatically in Figure 2.1:
Experimental work

Aims

Preliminary testing of the antifungal agents’ efficiency

Quantitative assessment of the drug delivery system

Qualitative assessment of the drug delivery system

Properties & characterisation of the drug delivery system

Chlorhexidine is a highly effective topical broad-spectrum agent against Candida species

Fungicidal amounts of antifungals are released from impregnated denture lining material for up to 28 days

Candidacidal effect of fluconazole and chlorhexidine released from acrylic polymer

Chlorhexidine impregnated PEM/THFM polymer exhibits superior activity to fluconazole against Candida albicans biofilm formation

Impregnation with antimicrobials challenge bonding properties and water sorption behaviour of an acrylic liner

The effect of antifungals incorporation on degree of conversion and colour stability of acrylic liner

Figure 2.1 A flow chart showing the work plan
CHAPTER 3

METHODOLOGIES
3.1 Introduction

Six *in vitro* studies were carried out using a range of standard techniques. The required paper format of the chapters (four to nine) did not allow an adequate description of the methodologies used. The methods and techniques employed are presented comprehensively in this chapter. Shear bonding, water absorption and colour stability are thoroughly detailed in their respective chapters.

3.2 Methods

3.2.1 Broth microdilution method

The broth microdilution (BMD) method as described in the CLSI M27-A3 document (Clinical Laboratory Standard Institute), used in this study, has been proven to produce comparable results to those obtained by the macrodilution method (Clinical Laboratory Standard Institute, 2008a). BMD has many advantages over macrodilution method; it is easier to perform, faster and more economic; therefore its use is highly preferable (Clinical Laboratory Standard Institute, 2008a).

Susceptibility testing generates a range of MIC values that can be categorised based on the *in vitro* response of an organism to an antimicrobial agent at levels corresponding to that achievable with a normally prescribed dose (Clinical Laboratory Standard Institute, 2008a). Antimicrobial susceptibility test interpretative categories are: 1) susceptible category: isolates are inhibited by normally attainable concentrations of the antimicrobial agent; 2) susceptible-dose dependent category: isolates are inhibited by a higher than normal dosage of the antimicrobial agent; 3) resistant category: isolates are not inhibited by the achievable
concentrations of the antimicrobial agent (Clinical Laboratory Standard Institute, 2008a).

In addition, quality control procedures and techniques were used in all microbiological investigations to ensure accuracy and reproducibility.

### 3.2.1.1 Preparation of drug dilution

Both pure fluconazole (FLUp; Pfizer, Kent, UK) and chlorhexidine (CHX) (Sigma-Aldrich, Dorset, UK) were tested. Stock solutions of FLUp (4096 mg/L) and CHX (10,000 mg/L) were prepared by dissolving 0.0123 and 0.0306 g in 3 ml DMSO (dimethyl sulphoxide; Sigma-Aldrich) respectively. The stock solutions were diluted in double strength RPMI-1640 with 2 % glucose in total (Sigma-Aldrich), by 1/2 for FLUp and 1/100 for CHX to give solutions of 2048 mg/L and 100 mg/L for FLUp and CHX respectively. A dilution range was prepared by serial double diluting the 4096 mg/L FLUp stock to 0.125 and 100 mg/L CHX stock to 0.2 mg/L (Figure 3.1a). The drug dilutions were dispensed into appropriate wells in microtitre assay plates (100 µl for each well) (Appleton Woods Limited, Birmingham, UK). Column 11 was the drug-free positive control (100 µl media and 100 µl inoculum) and column 12 was the inoculum-free negative control that acted as the medium sterility control (100 µl media and 100 µl sterile distilled water) (Figure 3.1b). Fluconazole from capsules (FLUc; Pfizer) was also tested for susceptibility and it was found that 2.5 times the quantity of FLUc compared to FLUp was required in order to give equivalent efficacy (Appendix II). For simplicity only CHX and FLUp is described in this chapter.
(a) Fluconazole dilution series

| Concentration | 4096 | 2048 | 1024 | 512 | 256 | 128 | 64 | 32 | 16 | 8 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 |

Chlorhexidine dilution series

| Concentration | 10,000 | 5000 | 2500 | 1250 | 625 | 312.5 | 156 | 78 | 39 | 20 | 100 | 50 | 25 | 12.5 | 6.25 | 3.125 | 1.56 | 0.78 | 0.39 | 0.2 |

(b) Figure 3.1 The diagram shows the fluconazole and chlorhexidine dilution series, the numbers highlighted in bold represent the concentrations used in the microtitre plates (a). A blank microtitre plate showing the wells where the prepared concentrations to be dispensed (b)
3.2.1.2 Preparation of inoculum

A single colony from the agar plate was picked off for each isolate to be tested using a sterile loop and suspended in 2 ml of sterile distilled water and vortexed. The turbidity of each suspension was examined visually and adjusted to equal 0.5 McFarland Standard (1-5 × 10^6 cells/ml). The inoculum was further diluted 1:10 to give a final suspension of 1-5 × 10^5 cells/ml to be used for colony counts. A further dilution by 1:100 in sterile water to give a suspension of 1-5 × 10^3 cells/ml was used in the assay and for colony counts and purity check.

3.2.1.3 Inoculation of assay plates

For each isolate 100 µl of the inoculum (1-5 × 10^3 cells/ml) was added into all the wells (1-11) in the appropriate row (one row for each isolate). The drug ranges were 0.125-2048 for FLU and 0.1-50 for CHX. Distilled sterile water (100 µl) was added to column 12. Then, all microtitre plates were incubated for 48 h at 37 °C with readings at 24 h and 48 h. For purity check a 10 µl loopful of 1-5 × 10^5 cells/ml suspensions as well as 10 µl of 1-5 × 10^3 cells/ml suspensions were spread onto half a blood agar and half a Sabouraud agar (Oxoid, Basingstoke, UK): all purity plates were incubated for 48 h at 37 °C.

3.2.1.4 Quality control

An isolate with a known MIC value to FLU was set up with each batch of test isolates. Candida krusei ATCC 6258 was used in all experiments: it has a MIC value to FLU of 16-64 mg/L. Purity plates, as described previously in § 3.2.1.3, ensured the quality control procedure.
3.2.1.5 Results interpretation

The MICs were read at an optical density (OD) of 490 nm by a spectrophotometer (BMG Labtech, Aylesbury, UK) at 24 h incubation (Figure 3.2). The plates were re-incubated for a further 24 h and MICs were re-read. For fluconazole, the minimum inhibitory concentration (MIC) was the lowest drug concentration that reduced the OD$_{490}$ by 50 % compared to the drug-free control. For chlorhexidine, the MIC was the lowest drug concentration that prevented any discernible growth (80 %). The purity plates were examined to ensure that yeast suspensions were not contaminated and were the correct concentration (purity plates of $1\times10^3$ cfu/ml should yield between 10 and 50 colonies).

Figure 3.2 Chlorhexidine microtitre plate showing clear wells where growth was inhibited and hazy wells with visible growth after $48$ h incubation
3.2.2 Bioassay method

Bioassay was used in this study to measure the concentration of CHX, FLU\textsubscript{p} and FLU\textsubscript{c} in the leachates of the impregnated discs by means of measuring and comparing the zone of inhibition of the tested samples with that of standards with known drug concentrations against susceptible microbes under standard condition.

3.2.2.1 Preparation of stock solutions and drug standards

A- Stock solution preparation

Stock solutions of chlorhexidine (A\textsubscript{chx}) and fluconazole (A\textsubscript{flu}) were prepared (1000 mg/L) by dissolving pure chlorhexidine (0.0031 g, 98 % purity) or fluconazole (0.0033 g, 90 %) powder in 3 ml of water in separate glass universals.

Stock solutions were diluted to obtain working concentrations:

- 100 mg/L - labelled as concentration B\textsubscript{flu} (100 µl of 1000 mg/L drug stock and 900 µl of water) for fluconazole
- 800 mg/L - labelled as concentration B\textsubscript{chx} (500 µl of 1000 mg/L drug stock and 150 µl of water) for chlorhexidine

B- Drug Standards preparation

The following standards were prepared: 6.25, 12.5, 25, 50, 100 mg/L for FLU and 100, 200, 400, 500, 600, 800 mg/L for CHX. These were prepared by adding sterile water to the concentrated stock solutions. These concentrations were prepared as follows:

Fluconazole Standards:

100 mg/L (B) add 100 µl of concentration A\textsubscript{flu} to 900 µl sterile water
50 mg/L (C) add 500 µl of concentration B to 500 µl sterile water
25 mg/L (D) add 500 µl of concentration C to 500 µl sterile water
12.5 mg/L (E) add 500 µl of concentration D to 500 µl sterile water
6.25 mg/L (F) add 500 µl of concentration E to 500 µl sterile water

Chlorhexidine Standards:
800 mg/L (B) add 500 µl of concentration \( A_{chx} \) to 125 µl sterile water
600 mg/L (C) add 500 µl of concentration \( A_{chx} \) to 333.5 µl sterile water
500 mg/L (D) add 500 µl of concentration \( A_{chx} \) to 500 µl sterile water
400 mg/L (E) add 500 µl of concentration B to 500 µl sterile water
200 mg/L (F) add 500 µl of concentration E to 500 µl sterile water
100 mg/L (G) add 500 µl of concentration F to 500 µl sterile water

3.2.2.2 Quality control

To ensure the accuracy of the procedure, control samples with known concentrations of CHX and FLU were placed on each bioassay plate. These are prepared as follows:

For fluconazole:
Internal standard 60 mg/L: solution \( B_{flu} \) is further diluted (400 µl of \( B_{flu} \) and 266.7 µl water) to give a 60 mg/L standard.
Internal standard 20 mg/L: solution \( B_{flu} \) is further diluted (400 µl of \( B_{flu} \) and 1.6 ml water) to give a 20 mg/L standard.

For chlorhexidine:
Internal standard 450 mg/L: stock solution \( A_{chx} \) is further diluted (100 µl drug stock and 122.22 µl water) to give a 450 mg/L standard.
Internal standard 250 mg/L: stock solution A<sub>chx</sub> is diluted (100 µl drug stock and 300 µl water) to give a 250 mg/L.

3.2.2.3 Preparation of inoculum

Suspensions of *C. kefyr* San Antonio and *C. albicans* (ATCC90028) strains were prepared separately in 7 ml of sterile distilled water to less than 0.5 McFarland Standard. The optical density of the suspension was adjusted to 0.026 and 0.04 (approximately 6 × 10<sup>5</sup> cells/ml) for *C. kefyr* and *C. albicans* respectively; of these suspensions, 5 ml was used as the inoculum.

3.2.2.4 Preparation of bioassay plates

Base agar (90 ml) was melted and cooled in a water bath for approximately 30 min (54 °C). Concentrated Yeast Nitrogen Base with Glucose (1 %) and Tri-sodium citrate (0.59 %) (YNBG + citrate) solution (10 ml) was also heated in the water bath (54 °C). Once the agar had cooled down, 10 ml of concentrated YNBG + citrate solution was added and mixed with the agar, then the pre-prepared 5 ml yeast inoculum was added. The bioassay plate (Appleton Woods Limited) (245 mm × 245 mm) was then poured and left to solidify for at least 30 min. To ensure that the bioassay plate was completely flat a levelling table was used. Thirty six wells of 8 mm diameter were cut out in the agar in a 6 × 6 pattern and left to dry for 1 h at 37 °C (Figure 3.3). Using a prepared template, 40 µl of each standard in triplicates, internal controls and the test specimen in duplicates were dispensed into the appropriate wells. After overnight incubation at 37 °C (approximately 18 h), the diameters of the zones of inhibition around each well were measured using digital callipers (Mitutoyo, Hampshire, UK), and recorded on the results template (Law et al., 1994).
3.2.2.5 Calculations and interpretation

The inhibition zones measurements of the drug standards were entered into the computer and were plotted against the logarithmic concentration of the standards as a regression curve; this was used as a standard graph (Figure 3.4), and was used to calculate the unknown drug concentrations in the test samples and in the internal controls. To pass the quality control requirement the internal controls had to be within 20% of their known values (Law et al., 1994).

Figure 3.3 Photographs of bioassay plates showing the wells where the leachates and the standards were dispensed (a), and the inhibition zones formed by the diluted leachates of chlorhexidine (b)
Figure 3.4 A representative view to show the known concentrations and the inhibition zone measurements (a), and the standard curve that was used to calculate the unknown drug concentrations [pink column in (a)]. Internal standards in this example passed the quality control requirement [within 20 % of their known concentrations (a)]
3.2.2.6 Validation

The bioassay was validated for linearity, precision, accuracy and reproducibility (FDA, 2001). Standard curves were judged linear if the coefficient of determination \( (r^2) \) using linear regression was >0.99 (Figure 3.4). The limit of detection was calculated as the smallest consistently detectable zone of inhibition (Lower Limit Of Quantification: LLOQ). Accuracy and variability (precision) of the method was assessed against five controls: LLOQ, very low, low, medium and high (6.25, 15, 40, 60 and 90 mg/L respectively) for fluconazole and (50, 100, 250, 400 and 500 mg/L respectively) for chlorhexidine. Intra-assay (within-batch) accuracy and precision were determined by analysing these same controls five times within one bioassay plate. Accuracy, (closeness of mean test results obtained by the method to the actual concentration value of the analyte) was calculated from the difference between the mean observed and nominal concentrations at a given level. Acceptance criteria dictated that the mean result must be within 20 % of the theoretical concentration for the LLOQ, and within 15 % for all other concentrations. As a measure of precision (the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous solution) an overall coefficient of variation (CV) less than 20 % was accepted for the LLOQ of this method. For all other concentrations, a CV less than 15 % was accepted (FDA, 2001).

3.2.3 Biofilm quantification methods

Biofilm quantification methods can be categorised into biofilm biomass assays (quantifying matrix and both living and dead cells) and viability assays (quantifying viable cells) (Peeters et al., 2008). XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) assay is a standard method for \textit{in vitro} biofilm
testing (Li et al., 2003). It is rapid and specific in nature because it measures the viability of cells within a mature biofilm (Peeters et al., 2008). The tetrazolium salt (XTT) is reduced to a water soluble formazan by cells, which is colorimetrically determined in the cell supernatant. The colorimetric change in the XTT reduction assay directly reflects the metabolic activity of the biofilm (Mowat et al., 2007).

Crystal violet (CV) staining method is another assay to quantify biofilms. It is a dye that binds to polysaccharides in the extracellular matrix and the negatively charged surface molecules (Li et al., 2003). However, it is not specific as it stains both living and dead cells, as well as matrix (Pitts et al., 2003).

The above methods have both been used in this research to quantify the biofilms that have been formed on control PEM/THFM discs, CHX and FLU impregnated PEM/THFM discs; they have been adapted from previous standard protocols (Ramage et al., 2001; Mowat et al., 2007) (described in details in § 3.2.3.2, § 3.2.3.3 and § 3.2.3.4).

### 3.2.3.1 Organisms and inoculum preparation

*C. albicans* ATCC 90028 strain was used in this study. A freshly grown isolate on Sabouraud agar was inoculated into 20 ml of yeast peptone dextrose (YPD) medium (1 % w/v yeast extract, 2 % w/v peptone, 2 % w/v dextrose, Oxoid) and incubated in an orbital shaker (100 rpm) overnight at 37 °C in order to obtain budding yeast phase. The cells were then harvested and washed in sterile phosphate buffered saline (PBS). Afterwards, cells were resuspended in RPMI-1640 supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS, Sigma-Aldrich) and the cell density was adjusted spectrophotometrically to $1.0 \times 10^6$ cells/ml to
produce the biofilm inoculum. This cell density is ideal for biofilm formation (Ramage et al., 2001).

### 3.2.3.2 Biofilm formation

All discs were prepared as described in Chapter 7, consisting of drug-free control discs, FLU and CHX impregnated discs. All discs were allocated into 24-well plates and 2 ml of the biofilm inoculum (1.0 × 10^6 cells/ml) was added to each well. The plates were carefully transferred and incubated at 37 °C. Biofilms were formed over a series of predetermined time points (2, 7, 14, 21 and 28 days). Ten replicates of discs impregnated with CHX were dedicated for each time point, from which five were treated with XTT to assess the metabolic activity and the other five were treated with crystal violet to assess the biofilm biomass (Figure 3.5). Similarly, ten FLU impregnated discs were tested for both methods at each time point. In addition, antifungal-free discs were also tested for both methods at each time point to serve as positive controls. The medium in all wells was replaced at each time point.

![Figure 3.5 Schematic diagram for the discs to be investigated by XTT (orange) and CV (purple) after different incubation periods (2, 7, 14, 21, 28 days)](image)
Both negative and positive controls were included. Negative controls consisted of discs incubated in RPMI without yeast inoculation (three replicates for each group CHX, FLUp, FLUc and the drug-free control), which were used as baseline measurements for their corresponding discs that incubated in RPMI with yeast inoculation. The positive controls were the drug-free acrylic discs with yeast inoculation.

3.2.3.3 XTT reduction assay

At each time point the incubated discs (drug-free control PEM/THFM, FLU and CHX impregnated PEM/THFM) were transferred carefully to a sterile 24-well plate and washed thoroughly three times with sterile PBS by repeated pipetting to remove the non-adherent cells. XTT (Sigma-Aldrich) was dissolved in PBS to produce a saturated solution at 0.5 g/L. Then the solution was filter sterilised through a 0.22 µm pore size filter, aliquoted and stored at -80 °C. Before each assay, an aliquot of XTT was thawed and menadione (10 mM prepared in acetone) was added to a final concentration of 1 µM. A 500 µl aliquot of XTT/menadione solution was added to each biofilm and to the positive and negative control wells, the latter to be used to measure background XTT reduction levels. The plates were covered with aluminium foil and transferred to 37 °C incubator for 3 h and then the colorimetric changes were measured using a microplate reader at a wavelength of 490 nm (Figure 3.6).
Figure 3.6 A photograph showing a microtitre plate with crystal violet stain (a) and XTT solution (b) collected for different biofilms

### 3.2.3.4 Biofilm biomass quantification

At each time interval the incubated discs (drug-free control PEM/THFM discs, FLU and CHX impregnated PEM/THFM discs) were transferred carefully to a sterile 24-well plate and washed thoroughly three times with sterile PBS by repeated pipetting to remove the non-adherent cells. The discs with the attached biofilm were air-dried and 500 µl of 0.5% (w/v) crystal violet solution (Sigma-Aldrich) was added for 5 min. The biofilms were then removed and carefully washed under running water until excess stain was removed. Afterwards, 500 µl of 95% ethanol (Sigma-Aldrich) was dispensed gently into the wells to completely solubilise the crystal violet and to destain the biofilms for 1 min. From each well, 100 µl was transferred to a clean 96-well microtitre plate and the absorbance of the collected solutions (intensity of the stain) was assessed using a microtitre plate reader at 584 nm (Figure 3.6). The biofilm biomass is proportional to the absorbance values.
3.2.4 Time-kill test

The time-kill test was used to evaluate the antimicrobial activity of the leachates of the impregnated PEM/THFM discs against selected candidal isolates. The advantages of this test over all other microbiological tests are that the rate and the extent of microbial killing can be estimated. The rate of activity of antimicrobial is an important factor to consider since it has a critical impact on the clinical outcome where rapid rate of activity is the most favourable (Lewis et al., 2002).

The leachates of the impregnated PEM/THFM were inoculated with a known population of Candida isolates for a specified period of exposure time at 37 °C. At selected time points aliquots were removed and serially diluted. Neat and diluted concentrations were plated onto agar and incubated at 37 °C for 48 h then colonies were counted. The results were calculated as a percentage reduction. The initial total viable count of the microbial population (control) was compared to the viable count with respect to each time point (May et al., 2000).

3.2.4.1 Disc preparation and incubation

This is explained extensively in Chapter 6. After discs incubation, leachates of control discs, CHX impregnated discs, pure fluconazole impregnated discs (FLUp) and fluconazole from capsules impregnated discs (FLUc) were obtained at 1, 3, 7, 14, 21 and 28 day time points.

3.2.4.2 Microorganisms and inoculum preparation

The isolates tested comprised three Candida isolates: one reference strain C. albicans ATCC 90028, and two clinical isolates C. albicans F/2511 and C. glabrata F/4023. These isolates were chosen for their varying degrees of susceptibility and resistance
to fluconazole where the former was susceptible and the other two isolates were resistant. The two latter isolates were obtained from the culture collection of the Mycology Reference Centre Manchester. Isolates were grown on Sabouraud agar for 48 h and then a couple of colonies were inoculated into 10 ml of distilled water and the cell density was adjusted spectrophotometrically to $1.0 \times 10^6$ cells/ml and further diluted to $1.0 \times 10^5$ cells/ml final cell density (working dilution).

3.2.4.3 Time-kill studies

Time-kill studies were carried out following standard protocols (Klepser et al., 1998). To detect the fungicidal activity of the leachates, 100 µl of working dilution was transferred to sterile tubes containing 900 µl of leachate of CHX, FLUp, FLUc and the drug-free control discs. The tubes were incubated at 37 °C and aliquots were removed at 1, 2, 4, 6, and 24 h post inoculation. Ten µl aliquots of the neat sample and two 10-fold serial dilutions were plated on Sabouraud agar and were incubated for 48 h at 37 °C to test viability. This was repeated for all different leachates collected at 3, 7, 14, 21, 28 days and against the three tested isolates. Viability counts of the working dilutions were used as controls. A fungicidal effect was defined as 100 % kill. Three hundred data points were collected for each of the six time intervals of leaching: 4 disc types, 5 replicates, 5 time-kill measurements and 3 Candida isolates.

3.2.4.4 Analysis

Viable counts for each agent at each leaching time point at each time-kill point were averaged and compared with the baseline reference and the control group. Kill curves were plotted with percentage of viability as a function of time for each isolate. The rate and extent of antifungal activity for each group were then compared among
leachates of different time intervals of leaching. A fungicidal effect was defined as a 100% reduction in the viable count.

3.2.5 Degree of conversion measurement

Degree of conversion (DC) was used in this study to measure the extent of polymerisation of PEM/THFM acrylic by measuring the percentage of aliphatic bonds (C=C) that have been converted into single bonds to form an extended network polymer (Stansbury and Dickens, 2001).

3.2.5.1 FTIR test

The fourier transform infrared (FTIR) spectrometer Avatar™ 360 was used to estimate the DC (Thermo Fisher Scientific, Surrey, UK). Micro-attenuated total reflectance was used (micro-ATR), which is a well established method (Niepraschk et al., 2007). The FTIR was used under the following conditions: 4000-500 cm\(^{-1}\) range, 4 cm\(^{-1}\) resolution, 32 scans coaddition. Initially, a background FTIR spectrum without a sample was first collected. Then the freshly mixed acrylic (dough stage) was distributed on the surface of the detector crystal of the FTIR spectroscope directly and covered to prevent light interference (Figure 3.7). A spectrum was recorded immediately for the uncured samples, and then the samples were left to set. Another FTIR spectrum was then collected for the cured samples 24 h later.
Figure 3.7 Photograph representing fourier transform infrared spectrophotometer connected to PC, showing the detector crystal against which the samples were distributed (arrow)

3.2.5.2 Results interpretation and analysis

The DC % was calculated from the aliphatic C=C peak at 1635 cm\(^{-1}\) and normalised against the carbonyl C=C peak at 1720 cm\(^{-1}\) (Figure 3.8) according to the following formula:

\[
\text{Eq.}(3.1)
\]

\[
\text{Aliphatic}_{\text{cured}} = \text{absorption peak at 1635 cm}^{-1}\text{ of the cured specimen.}
\]
\[
\text{Carbonyl}_{\text{cured}} = \text{absorption peak at 1720 cm}^{-1}\text{ of the cured specimen.}
\]
\[
\text{Aliphatic}_{\text{uncured}} = \text{absorption peak at 1635 cm}^{-1}\text{ of the uncured specimen.}
\]
\[
\text{Carbonyl}_{\text{uncured}} = \text{absorption peak at 1720 cm}^{-1}\text{ of the uncured specimen}
\]
Figure 3.8 Full FTIR spectrum of the uncured specimen from 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) and a closer view of the area of interest (spectrum from 1790 cm\(^{-1}\) to 1505 cm\(^{-1}\)) (a), FTIR spectrum of the cured specimen and a closer view of the area of interest (spectrum from 1790 cm\(^{-1}\) to 1505 cm\(^{-1}\)) (b)

3.2.6 UV Spectrophotometry

One of the methods that were used in this study to quantify the amount of the released drugs in the leachates of the impregnated PEM/THFM acrylic discs was UV spectrophotometry (Shimadzu, Kyoto, Japan) at 220 nm. Triplicate 10-fold
dilution series of the leachates were analysed at 1, 3, 7, 14, 21 and 28 days. The absorbance readings of the drug-free control leachates were used as a reference. The unknown concentrations of drug in each leachate were calculated using standard curves of known concentrations of chlorhexidine (Table 3.1 and Figure 3.9) and fluconazole (Table 3.2 and Figure 3.10).

**Table 3.1 Absorbance values for different known concentrations of chlorhexidine**

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance</th>
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</thead>
<tbody>
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</tr>
<tr>
<td>CHXA</td>
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</tr>
<tr>
<td>CHXB</td>
<td>20</td>
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</tr>
<tr>
<td>CHXC</td>
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</tr>
<tr>
<td>CHXD</td>
<td>50</td>
<td>3.28601</td>
</tr>
<tr>
<td>CHXE</td>
<td>60</td>
<td>3.91412</td>
</tr>
<tr>
<td>CHXF</td>
<td>75</td>
<td>4.81738</td>
</tr>
</tbody>
</table>

**Figure 3.9 Chlorhexidine standard curve**
Table 3.2 Absorbance values for different known concentrations of fluconazole

<table>
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<th>Sample ID</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
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<td>0</td>
</tr>
<tr>
<td>FLUA</td>
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</tr>
<tr>
<td>FLUB</td>
<td>20</td>
<td>0.72655</td>
</tr>
<tr>
<td>FLUC</td>
<td>30</td>
<td>1.02223</td>
</tr>
<tr>
<td>FLUD</td>
<td>40</td>
<td>1.36870</td>
</tr>
<tr>
<td>FLUE</td>
<td>50</td>
<td>1.71835</td>
</tr>
<tr>
<td>FLUF</td>
<td>60</td>
<td>1.99432</td>
</tr>
<tr>
<td>FLUG</td>
<td>75</td>
<td>2.41435</td>
</tr>
<tr>
<td>FLUH</td>
<td>100</td>
<td>3.18853</td>
</tr>
</tbody>
</table>

Figure 3.10 Fluconazole standard curve
CHAPTER 4

Chlorhexidine is a highly effective topical broad-spectrum agent against Candida species

Nesreen Salim, Caroline Moore, Nick Silikas, Julian Satterthwaite, Riina Rautemaa

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4.1 Abstract

The objective of this study was to compare the in vitro antifungal activity of chlorhexidine and fluconazole against Candida isolates comprising eight different species associated with oral candidosis. A broth microdilution (BMD) method as described in the Clinical Laboratory Standard Institute protocol M27-A3 was used for determining susceptibility. A total of 79 clinical isolates and reference strains belonging to eight different Candida species were tested. The MIC was the lowest drug concentration that reduced growth by 50 % for fluconazole at 48 h and 80 % for chlorhexidine at 24 h and 48 h. The geometric mean MIC at 48 h for chlorhexidine was 3.03 (0.78-6.25 mg/L) and for fluconazole 19.12 (≤0.125-256 mg/L). Of the isolates, 14 of 79 (18 %) were resistant to fluconazole (MIC ≥64 mg/L). All isolates were effectively inhibited by ≤6.25 mg/L of chlorhexidine and no cross-resistance between chlorhexidine and fluconazole was detected (r=0.039, P=0.733). CLSI M27-A3 methodology proved to provide reproducible results with clear end-points for chlorhexidine. In conclusion, the findings showed that chlorhexidine has excellent broad-spectrum antifungal activity in vitro. It was effective at concentrations detected in saliva when using standard dosing regimens. Moreover, no cross-resistance was detected between chlorhexidine and fluconazole, even among Candida species highly resistant to fluconazole.

Key words: fluconazole, chlorhexidine, Candida, susceptibility, minimum inhibitory concentration.
4.2 Introduction

Being the disease of the diseased, oral candidosis predominantly affects immunosuppressed and medically compromised patients (Coco et al., 2008). In these high-risk patients the oral cavity may provide a source for *Candida* causing systemic infection (Rautemaa and Ramage, 2011). Oral candidosis has become a significant challenge in patients with persisting risk factors and a recurrent need for antifungal treatment. In particular, repeated courses of fluconazole have been shown to form a risk for persistent colonisation with microbiologically and clinically resistant *Candida* (Johnson et al., 1995; Rautemaa and Ramage, 2011). Oral candidosis is a mixed multi-species candidal-bacterial biofilm infection which provides multiple challenges for its management (Ramage et al., 2005). The biofilm lifestyle is commonly associated with poor drug penetration and antimicrobial recalcitrance as well as a risk of development of resistance (Cowen et al., 2002; Ramage et al., 2005).

A number of antifungal agents are available for the management of fungal infections (Samaranayake et al., 2009); however, the choice of antifungals suitable for the treatment of oral candidosis is limited (Rautemaa and Ramage, 2011). Fluconazole is a widely used systemic antifungal agent which is well tolerated with low toxicity and mild side effects (Samaranayake et al., 2009) although in elderly patients with reduced saliva production, there is a risk of low drug levels in the oral cavity and emergence of resistance (Siikala et al., 2010). Moreover, non-*albicans* species *Candida* such as *C. glabrata* and *C. krusei*, are intrinsically resistant to fluconazole, and are common causes of oral candidosis (Bagg et al., 2003; Coco et al., 2008). In addition, fluconazole’s penetration into candidal biofilm is poor, leading to low drug concentrations which again has a potential risk for selection and development of
resistant strains (Ramage et al., 2002a). Nystatin is a highly effective topical antifungal with few drug interactions; however, its four-times daily dosage is a significant challenge for patient compliance (Blomgren et al., 1998; Su et al., 2008). Echinocandins are highly effective agents against Candida and Candida biofilms (Ramage et al., 2002b); however, their availability only as an intravenous formulations and high cost negate their use for the treatment of oral candidosis (Rautemaa and Ramage, 2011).

Chlorhexidine has been used as an adjunctive therapeutic option for topical use due to its broad spectrum antimicrobial efficiency (Ramage et al., 2011). It is effective at low concentrations and has unique substantivity extending its therapeutic effect in the oral cavity due to its high adsorption capacity such that it can be retained in the oral cavity for long periods (up to 12 h) (Ellepola and Samaranayake, 2001; Tomas et al., 2010): consequently, less frequent dosing can be used (Ellepola and Samaranayake, 2000b, 2001). The mode of action of chlorhexidine on Candida is still unclear but it has been suggested that it inhibits cell wall synthesis by binding to negatively charged groups in the candidal cell wall followed by intracellular material leakage and cell death (Hiom et al., 1992). It appears to inhibit candidal replication and the adhesion of Candida to epithelial cells and denture surfaces, all being crucial prerequisites for fungal infection (Ellepola and Samaranayake, 2001). Chlorhexidine has been described as having significant activity against C. albicans in vitro, but fewer data exist for Candida species other than C. albicans, such as C. glabrata, C. tropicalis and C. krusei (Hiom et al., 1992; Ramage et al., 2011). It has also been shown to have superior efficacy against Candida biofilms compared to fluconazole in vitro and in vivo (Thurmond et al., 1991;
Furthermore, it can be used to impregnate denture liners to act as a long-term self-release drug carrier (Salim et al., 2012a).

There is a clear clinical and microbiological need for the evaluation of the in vitro antifungal activity of chlorhexidine. The present study aimed to investigate the antifungal activity of chlorhexidine against a panel of isolates belonging to a number of different Candida species commonly isolated from patients with oral candidosis, and to compare its activity against fluconazole. The null hypotheses were, firstly, that chlorhexidine is effective against a broad spectrum of Candida species and, secondly, that it has a comparable activity to fluconazole at levels seen in saliva.
4.3 Materials & Methods

Organisms and Media

A total of 79 Candida isolates belonging to eight different species, comprising 76 clinical isolates and three reference strains were tested against chlorhexidine (CHX) and fluconazole (FLU). The clinical isolates were obtained from the culture collection of the Mycology Reference Centre (Manchester, UK) and were predominantly obtained from mucocutaneous and haematogenous sources from patients, including those with immunodeficiency, candidaemia and tissue-invasive disease. American Type Culture Collection (ATCC) strains C. albicans ATCC 90028, C. krusei ATCC 6258 and C. tropicalis ATCC 750 were used as reference strains (Clinical Laboratory Standard Institute, 2008b). Isolates were identified by standard biochemical methods including CHROMagar Candida medium (CHROMagar, Paris, France), API ID32C (Bio-Merieux, Lyon, France) assimilation tests, and C. dubliniensis agglutination test (Bichro-Dubli Fumouze, Fumouze Diagnostics, France). The isolates were stored at -80 °C and each isolate was plated twice on Sabouraud agar (Oxoid, Basingstoke, UK) and incubated at 37 °C for 48 h before use to check viability and purity. A total of 32 C. albicans, 13 C. glabrata, 10 C. dubliniensis, 6 C. parapsilosis, 6 C. guilliermondii, 6 C. tropicalis, 5 C. krusei, and 1 C. kefyr were tested. RPMI-1640 with 2 % glucose, buffered with morpholinopropanesulfonic acid (MOPS; Sigma-Aldrich, Dorset, UK) and adjusted to pH 7.0 was used as growth medium for FLU and CHX. The reproducibility of the method was evaluated by retesting 20 % of randomly selected isolates (16/79) against each drug. The same batch of medium was used throughout the study, including reproducibility studies.
Susceptibility Testing and Antifungal Agents

The broth microdilution (BMD) method as described in the CLSI M27-A3 document was used for determining susceptibility (Clinical Laboratory Standard Institute, 2008a). Fluconazole (Pfizer, Kent, UK) and chlorhexidine (Sigma-Aldrich) were obtained in pure powder form from their respective manufacturers. Briefly, 2-fold dilution series of FLU (0.125-2048 mg/L) and CHX (0.1-50 mg/L) were prepared in sterile distilled water and an inoculum of 1 × 10^3 cells/ml was used. After incubation at 37 °C, the growth in each well was measured by spectrophotometry (BMG Labtech, Aylesbury, UK) at 490 nm. For FLU, the MIC was the lowest drug concentration that reduced the OD_{490} by 50 % at 48 h compared to the drug-free control. The CLSI standard breakpoints for fluconazole were used for susceptibility interpretation (Clinical Laboratory Standard Institute, 2008b; Versalovic, 2011). The isolates were designated susceptible (S), susceptible-dose dependent (S-DD), or resistant (R) based on their MICs and according to CLSI standard (Clinical Laboratory Standard Institute, 2008b; Versalovic, 2011). For CHX, the MIC was the lowest drug concentration that reduced the OD_{490} by 80 % at 24 h and 48 h compared to the drug-free control. Sabouraud agar and blood agar plates were inoculated with 10 μl of each organism suspension to check the viable count and culture purity. Geometric means (GM) and ranges were calculated.

Statistical Analysis

The SPSS statistical package 18.0 (SPSS Inc., Chicago, Illinois, USA) was used to analyse all data. A Kruskal-Wallis test was used to verify differences in susceptibility between species against CHX at P≤0.05 with post hoc Mann–Whitney U tests. A Wilcoxon test was performed to compare MIC values at 24 h and 48 h for each
species against CHX. The correlation between the antifungal activity of CHX and FLU against *Candida* species was evaluated using spearman's rho ($r_s$). The ranking was used to establish if the correlation coefficient was significantly different from zero. The significance level was determined at $P \leq 0.05$. 
4.4 Results

The GM MIC for CHX for all *Candida* isolates was 2.22 mg/L at 24 h and 3.03 mg/L at 48 h (Table 4.1) and MIC90 was 6.25 mg/L (0.78-6.25 mg/L) at 48 h. The MIC at 48 h was significantly higher than at 24 h for five species: *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. guilliermondii* and *C. krusei* ($P \leq 0.05$). For three species the incubation time did not have an impact on the MIC, *C. dubliniensis*, *C. tropicalis* and *C. kefyr* ($P > 0.05$) although only a small number of isolates were tested for some species. The GM MIC for *C. albicans* and *C. glabrata* was significantly higher than that detected for all other species at 48 h read ($P \leq 0.05$).

Table 4.1 Chlorhexidine geometric mean MIC results for 79 *Candida* isolates belonging to 8 different species at 24 h and 48 h incubation

<table>
<thead>
<tr>
<th>Candida species</th>
<th>MIC at 24 h (mg/L)</th>
<th>MIC at 48 h (mg/L)</th>
<th>Significancea</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. albicans</em> (32)</td>
<td>4.05</td>
<td>5.03</td>
<td>0.001</td>
</tr>
<tr>
<td><em>C. glabrata</em> (13)</td>
<td>3.13</td>
<td>4.78</td>
<td>0.005</td>
</tr>
<tr>
<td><em>C. dubliniensis</em> (10)</td>
<td>3.13</td>
<td>3.13</td>
<td>1.0</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (6)</td>
<td>1.56</td>
<td>3.13</td>
<td>0.014</td>
</tr>
<tr>
<td><em>C. guilliermondii</em> (6)</td>
<td>0.78</td>
<td>1.10</td>
<td>0.046</td>
</tr>
<tr>
<td><em>C. tropicalis</em> (6)</td>
<td>2.78</td>
<td>3.13</td>
<td>0.317</td>
</tr>
<tr>
<td><em>C. krusei</em> (5)</td>
<td>1.56</td>
<td>3.13</td>
<td>0.025</td>
</tr>
<tr>
<td><em>C. kefyr</em> (1)</td>
<td>0.78</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>All isolates (79)</td>
<td>2.22</td>
<td>3.03</td>
<td></td>
</tr>
</tbody>
</table>

a Differences between MICs at 24 h and 48 h were tested by Wilcoxon test

The cumulative percentage of isolates for each species of *Candida* inhibited at each concentration of CHX and FLU throughout the broth microdilution (BMD) series is presented in Tables 4.2A and 4.2B, respectively. CHX demonstrated antifungal
activity against all tested isolates with low MIC values ranging from 0.78 to 6.25 mg/L and trailing end points with CHX were usually not encountered and 100 % of the tested isolates were inhibited by 6.25 mg/L of CHX. For FLU the MIC values ranged from ≤0.125 to 256 mg/L with an overall GM MIC of 19.12 mg/L and MIC90 of 128 mg/L (0.125-256 mg/L). Of all isolates 18 % were resistant to FLU (MIC ≥ 64 mg/L) (Table 4.2B). FLU was highly active against C. dubliniensis, C. parapsilosis, C. guilliermondii and C. kefyr (all had a MIC ≤8 mg/L) but less active against C. krusei, C. glabrata and C. tropicalis (0 %, 15 % and 16 % had a MIC ≤8 mg/L, respectively). Of the C. albicans isolates, 90 % had a MIC ≤8 mg/L. C. krusei was highly resistant (100 % had a MIC ≥64). None of the FLU-resistant isolates were resistant to clinically relevant levels of CHX and the MIC values for FLU did not correlate with those for CHX (r²=0.039, P=0.733), whereby no cross-resistance was detected in vitro (Figure 4.1). The lack of correlation of CHX and FLU MICs is demonstrated in Figure 4.1. The GM MIC of all Candida isolates for FLU was over 6-fold that of CHX and CHX showed minimal variation: MICs for CHX were within 4 dilutions whereas MICs for FLU were within 12 dilutions (Figure 4.1).
Table 4.2  Cumulative percentage of isolates for each species of *Candida* inhibited at each concentration in broth microdilution series

A. Susceptibility of *Candida* species to chlorhexidine by MIC at 80% after 48 h

<table>
<thead>
<tr>
<th>Species</th>
<th>Cumulative percentage of strains at MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(No. of isolates) 0.09 0.19 0.39 0.78 1.56 3.125 6.25 12.5 25 50</td>
</tr>
<tr>
<td><em>C. albicans</em> (32)</td>
<td></td>
</tr>
<tr>
<td><em>C. glabrata</em> (13)</td>
<td></td>
</tr>
<tr>
<td><em>C. dubliniensis</em> (10)</td>
<td></td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (6)</td>
<td></td>
</tr>
<tr>
<td><em>C. Guilliermondii</em> (6)</td>
<td></td>
</tr>
<tr>
<td><em>C. tropicalis</em> (6)</td>
<td></td>
</tr>
<tr>
<td><em>C. krusei</em> (5)</td>
<td></td>
</tr>
<tr>
<td><em>C. kefyr</em> (1)</td>
<td></td>
</tr>
<tr>
<td>All isolates (79)</td>
<td>4 9 62 100</td>
</tr>
</tbody>
</table>

*Broth microdilution MICs determined using CLSI M27-A3 (Clinical Laboratory Standard Institute, 2008a)*
### B. Susceptibility of Candida species to fluconazole by MIC at 50% after 48 h

<table>
<thead>
<tr>
<th>Species</th>
<th>Cumulative percentage of strains at MIC&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(No. of isolates)</td>
</tr>
<tr>
<td>C. albicans (32)</td>
<td></td>
</tr>
<tr>
<td>C. glabrata (13)</td>
<td></td>
</tr>
<tr>
<td>C. dubliniensis (10)</td>
<td></td>
</tr>
<tr>
<td>C. parapsilosis (6)</td>
<td></td>
</tr>
<tr>
<td>C. guilliermondii (6)</td>
<td></td>
</tr>
<tr>
<td>C. tropicalis (6)</td>
<td></td>
</tr>
<tr>
<td>C. krusei (5)</td>
<td></td>
</tr>
<tr>
<td>C. kefyr (1)</td>
<td></td>
</tr>
</tbody>
</table>

*Broth microdilution MICs determined using CLSI M27-A3 (Clinical Laboratory Standard Institute, 2008a)

<sup>b</sup>SMIC, ≤ 8 mg/L; S-DD MIC, 16 to 32 mg/L (grey shaded area); R MIC, ≥64 mg/L (Clinical Laboratory Standard Institute, 2008b; Versalovic, 2011)
Figure 4.1  Scatterplot showing the relationship between fluconazole and chlorhexidine MICs obtained with 79 isolates of *Candida* species ($r_s$=0.039, $P$=0.733). Each number in the graph represents the number of isolates with a particular chlorhexidine MIC value and the corresponding fluconazole MIC value.

The MIC of all *Candida* isolates was lower than the peak CHX concentration normally detected in saliva (154 mg/L) (Bonesvoll and Gjermo, 1978) using normal dosing regimens (twice daily rinsing; Figure 4.2). The MIC of all *Candida* isolates was lower than the trough CHX concentration normally detected in saliva. The peak saliva concentration of FLU when normal dosing regimens of a single oral dose of 100 mg of FLU are used (Force and Nahata, 1995) exceeds the MICs of 57 % of all tested *Candida* isolates, 81 % of *C. albicans* and none of *C. glabrata* isolates. The MIC of 48 % of all *Candida* isolates was higher than the trough FLU concentration normally detected in saliva. This percentage represents 10 % *C. dubliniensis*, 19 % *C. albicans*, 33 % *C. parapsilosis*, 84 % *C. tropicalis* and 100 % *C. krusei* and 100 % of *C. glabrata* isolates tested.
Figure 4.2  Distribution of MICs (mg/L) and the GM means (-----) of 79 Candida isolates comprising 8 Candida species for chlorhexidine (a) and fluconazole (b). The hatched area represents the normal salivary concentration for each agent with normal dosing regimens, twice chlorhexidine rinsing daily and single oral dose of 100 mg of fluconazole (Bonesvoll and Gjermo, 1978; Force and Nahata, 1995)
The MICs for *C. albicans* ATCC 90028, *C. krusei* ATCC 6258 and *C. tropicalis* ATCC 750 tested as part of quality control were within the recommended ranges. All 16 isolates when re-tested against FLU and CHX produced either identical results or gave a result that differed by one two-fold dilution only.
4.5 Discussion

The current study showed that chlorhexidine has an excellent antifungal *in vitro* activity against a broad spectrum of *Candida* species. It was effective against all *Candida* species at concentrations detected in saliva when using standard dosing regimens. These findings are in line with previous limited data (Giuliana *et al.*, 1999). The susceptibility to chlorhexidine was compared to that of fluconazole for a wide range of *Candida* species. Of the tested isolates, 12% were dose-dependent and 18% resistant to fluconazole. Interestingly, chlorhexidine was effective also against those *Candida* isolates with reduced susceptibility to fluconazole and no cross-resistance was detected. This has not been reported previously. Consequently the first hypothesis, that chlorhexidine is effective against a broad spectrum of *Candida* species, was accepted but the second hypothesis was rejected as its efficacy was found to be superior to that of fluconazole.

*C. albicans* and *C. glabrata* appeared to be less susceptible to chlorhexidine compared to the other species tested. Nevertheless, their MICs were lower than the mean trough salivary chlorhexidine levels when a standard twice-daily rinsing regimen is used (Bonesvoll and Gjermo, 1978). The highest MIC recorded was 6.25 mg/L and the mean level of chlorhexidine in saliva at 12 h after rinsing has been reported to be 20 mg/L (Bonesvoll and Gjermo, 1978). The highest MICs were also 10-fold lower than the break point of 70 mg/L previously suggested for oral bacteria (Slots *et al.*, 1991). These *in vitro* results are consistent with the results of a previous *in vivo* study which showed a good response of oral candidosis to chlorhexidine treatment (Lal *et al.*, 1992).
Fluconazole showed a considerable antifungal activity against 70 % of the tested isolates. However, some or all *C. krusei, C. tropicalis* and *C. glabrata* showed resistance. Fluconazole break points for *C. albicans, C. tropicalis, C. parapsilosis, C. glabrata* and *C. krusei* applied in this study are presently subject to revision and the suggested new break points would designate an even higher proportion of the isolates as resistant (Pfaller *et al.*, 2010). Importantly, the MICs of 43 % of the tested *Candida* isolates and 19 % of *C. albicans* and 100 % of *C. glabrata* were higher than the mean peak saliva concentrations of fluconazole with a standard dosing regimen of once daily oral 100 mg of fluconazole (Force and Nahata, 1995). At 24 h, the salivary level of fluconazole falls below the MICs of 48 % of the tested isolates (Force and Nahata, 1995). This is of clinical importance, since the risk for development of antifungal resistance in *Candida* species has been linked to low drug levels (Rautemaa and Ramage, 2011). Moreover, even the highest achievable salivary fluconazole concentrations are lower than the MICs of all *C. glabrata* isolates. *C. glabrata* is an important cause for oral candidosis and particularly for denture-induced stomatitis (Coco *et al.*, 2008).

There is no established *in vitro* method for testing susceptibility of *Candida* to chlorhexidine. This study confirms that the CLSI M27-A3 methodology is suitable and provides reproducible results with clear end-points. The results of this study also showed a significant increase in MIC readings from 24 h to 48 h for most species. This is in line with the CLSI standard which generally recommends the final reading at 48 h for all drugs. However, the CLSI standard provides 24 h reading break points for *C. parapsilosis* and *C. krusei* whereas our results show a significant increase for these species after 24 h. On the other hand, in our study the difference between the
two readings for *C. dubliniensis* and *C. tropicalis* was not significant. The relationship between *in vitro* MIC results and clinical outcome is complex (Cowen *et al.*, 2002). A number of patient variables such as the immune status of the patient and the chronicity of the fungal infection may affect the response, and that the MIC alone and the MIC/tissue level ratio does not predict the treatment success. For example, it is possible that fluconazole levels reached in oral epithelium may be sufficient to inhibit the invasion of *Candida* into underlying tissues and ease the symptoms despite low salivary levels and no change in the candidal load in the oral cavity.
4.6 Conclusions

Our findings demonstrate that chlorhexidine has excellent antifungal efficacy against a broad range of *Candida* species and is superior to that of fluconazole. It was effective at concentrations detected in saliva when using standard dosing regimens. Moreover, no cross-resistance was detected between chlorhexidine and fluconazole, even among *Candida* species highly resistant to fluconazole. Our work, together with the previous data showing excellent activity of chlorhexidine against candidal biofilms, reinforces the use of chlorhexidine for the treatment of oral candidosis (Ramage *et al.*, 2011). It could be used as alternative to commonly used antifungal drugs or as an adjunct therapy especially in complicated recurrent oral fungal infections.
4.7 Funding

This work was supported in part by The University of Jordan (NS). The authors have no conflicts of interest to declare.
CHAPTER 5

Fungicidal amounts of antifungals are released from impregnated denture lining material for up to 28 days

Nesreen Salim, Caroline Moore, Nick Silikas, Julian D.Satterthwaite, Riina Rautemaa

Journal of Dentistry. 2011, 40:506-51
5.1 Abstract

Objectives: The aim of this study was to investigate the efficacy of a polymeric delivery system impregnated with chlorhexidine or fluconazole against Candida species.

Methods: Self-cure poly (ethyl methacrylate) and tetrahydrofurfuryl methacrylate (PEM/THFM) discs impregnated with pure fluconazole substance (FLUp), fluconazole powder from capsules (FLUc) or chlorhexidine powder (CHX) were incubated in water for up to 28 days at 37 °C. The water was replaced at 24 h and 3, 7, 14, 21, 28 days. The amount of released drugs and antifungal activity of the leachates was measured by bioassay. The minimal inhibitory concentration (MIC) of each drug for 46 Candida isolates was determined and compared to the released concentrations.

Results: A total of 53.0 % of CHX, 38.5 % of FLUc and 13.2 % of FLUp impregnated into the discs was leached during the 28-day incubation. Of the total amount leached, 71.8 % of CHX, 75.1 % of FLUc and 70.5 % of FLUp was released during the first week of incubation. Antifungal activity was confirmed for up to 28 days.

Conclusion: Both chlorhexidine and fluconazole become readily leached from PEM/THFM polymer up to 4 weeks and that the polymerisation of the acrylic does not affect the antimicrobial activity of the agents. Importantly, the amount of drugs released exceeded the MICs of most isolates also during the fourth week of incubation.

Clinical significance: These findings indicate the feasibility of this treatment modality for oral candidal infections, especially denture-induced stomatitis. But further in vivo work is warranted to determine clinical relevance and applicability.
5.2 Introduction

*Candida*-associated denture-induced stomatitis is a common disease in elderly denture wearers with a prevalence of 45-70 % (Figueiral *et al.*, 2007; Dagistan *et al.*, 2009). It is a multifactorial disease and *Candida albicans* is the principal causative agent. Other *Candida* species such as *C. glabrata* are commonly identified especially in medically compromised patients (Coco *et al.*, 2008; Rautemaa and Ramage, 2011). Many therapeutic modalities are available ranging from denture disinfection to systemic antifungal therapy (Webb *et al.*, 1998c; Uludamar *et al.*, 2011). Despite this the recurrence rate of denture-induced stomatitis is considerably high (Lal *et al.*, 1992; Cross *et al.*, 2004). It has been suggested that this is due to poor access of the antifungals onto the fitting surface, their poor penetration into the microbial biofilm on the porous denture material, as well as their rapid clearance by saliva and tongue movements (Addy and Fugit, 1989; Cross *et al.*, 2004).

A drug commonly used for the treatment of oral candidosis is systemic fluconazole (Rautemaa and Ramage, 2011). However, in elderly patients with reduced saliva production, therapeutic levels form a risk for emergence of microbiological and clinical resistance (Siikala *et al.*, 2010). Topical antifungals are effective but require daily compliance for frequent dosing (Geerts *et al.*, 2008). Chlorhexidine is a widely used disinfectant with a remarkable antifungal, antibacterial and anti-biofilm potency (Ramage *et al.*, 2011). Significantly, emergence of resistance has not been observed with chlorhexidine (Figueiral *et al.*, 2007; Dagistan *et al.*, 2009).

Local drug carriers have been suggested to prolong the efficiency of oral treatment as ideal therapeutic drug levels can be maintained at the site of infection over the
required period by release of the drug at a predetermined controlled rate (Douglas, 1977; Geerts et al., 2008). Drug carriers are also convenient for patients as they do not require compliance to frequent application regimes. In addition, direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug-drug interactions. Favourable results with incorporation of different antifungal agents in polymeric systems used in dentures have been reported (Addy, 1981; Geerts et al., 2008; Amin et al., 2009). Chow et al (1999) have shown that denture liner containing nystatin, fluconazole or itraconazole has detectable antifungal activity. It has also been demonstrated that mixing chlorhexidine, fluconazole, nystatin or clotrimazole into a soft lining material results in inhibition of candidal growth, demonstrating the release of the drugs from the liner (Schneid, 1992). Ryalat et al (2011) demonstrated that chlorhexidine could be released steadily from self-cured poly (methyl methacrylate) acrylic throughout the 28-day test period. Truhlar et al (1994) incorporated nystatin with two different soft liners and demonstrated a constant fungicidal activity up to 14 days. A self-cured poly (ethyl methacrylate)/tetrahyrofurfuryl methacrylate (PEM/THFM) polymeric system has been shown to have good biocompatibility, unique water uptake properties and potential for a drug carrier (Pearson et al., 1986; Patel and Braden, 1991b; Patel et al., 2001). A dose-dependent release pattern of chlorhexidine impregnated into PEM/THFM has been demonstrated with excellent inhibition of candidal growth (Patel et al., 2001). Moreover, exceptional water uptake characteristic of PEM/THFM acrylic resin is considered an important attribute to enhance drug delivery of the acrylic system (Riggs et al., 2000).
Various techniques have been used to measure the release of the antifungal drugs from the drug carriers (Addy, 1981; Wilson and Wilson, 1993). Spectrophotometry is easy to use and provides reliable results of drug concentrations in solutions (Wilson and Wilson, 1993), whereas HPLC (High Performance Liquid Chromatography) is technically more demanding but is more accurate (Amin et al., 2009). However, neither of these methods measures the bioavailability and activity of the released drug. Bioassay is a well known technique used widely in microbiological investigations (Law et al., 1994). It is commonly employed to measure the drug concentrations in plasma samples from patients (Speller, 1984; Kim et al., 2003) and provides both quantitative and qualitative information about the released drugs (Andes et al., 2009).

The aim of the present study was to investigate the utility of PEM/THFM as a delivery system for a sustained release of antifungals using a bioassay technique. This system has previously been used as a drug carrier for chlorhexidine but its applicability as a carrier for fluconazole has not been previously demonstrated (Patel et al., 2001; Gong et al., 2007). Furthermore, the bioactivity of the leached drugs has not been previously analysed using a qualitative and quantitative method such as bioassay. The hypothesis was that PEM/THFM system could be used for the effective local delivery of fluconazole and chlorhexidine in the treatment of denture-induced stomatitis.
5.3 Materials and methods

Study Design

Five parallel poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) discs were prepared by replacing a proportion of the PEM by pure fluconazole (FLUp), fluconazole powder from capsules (FLUc) or chlorhexidine (CHX). Five control discs were prepared following manufacturer's instructions. All discs were incubated in sterile distilled water for up to 28 days at 37 °C. The water was collected and replaced at 24 h and 3, 7, 14, 21, 28 days. The amount of released drugs and antifungal activity of the leachates was measured by bioassay. The minimal inhibitory concentration (MIC) of each drug for a total of 46 Candida isolates was determined and compared to the released concentrations.

Preparation of PEM/THFM Discs

For the control discs, 1 g of PEM (Lucite International, Durham, UK) and 0.6 ml of THFM (Sigma-Aldrich, Dorset, UK) were mixed. For the impregnated discs, 10 % (w/w) of the PEM was replaced with CHX (Sigma-Aldrich), 10 % (w/w) with FLUp (Sigma-Aldrich) or 25 % (w/w) with FLUc (Pfizer, Kent, UK). Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25 % of it is equivalent to 10 % of pure fluconazole, as confirmed by bioassay and MIC testing. The drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed. All mixtures were packed into disc shaped steel moulds (40 mm diameter and 0.5 mm height) and allowed to cure for 15 min.
**Disc Incubation**

Discs were soaked individually in 20 ml of sterile distilled water at 37 °C in tightly sealed 100 ml sterile plastic containers. The containers were gently shaken 3 times daily throughout the experiment to simulate the clinical situation. Drug concentrations within the leachates were detected using a bioassay method. The leachates of the control discs (acrylic without drug) were used as negative controls.

**Bioassay**

Bioassay was performed following a standard protocol (Law et al., 1994). A suspension of *C. kefyr* San Antonio strain or *C. albicans* (ATCC90028) was mixed into melted agar together with yeast nitrogen base with glucose (1 %) and tri-sodium citrate (0.59 %) (YNBG + citrate) solution and poured into a plate (245 mm × 245 mm). Wells of 8 mm diameter were cut out in the agar. Two-fold dilution series of pure fluconazole and chlorhexidine were prepared and these known concentrations were used as drug standards and internal controls (Law et al., 1994).

Triplicates of drug standards (FLUp and CHX), duplicates of internal controls (FLUp and CHX) and duplicates of the leachate test specimens were dispensed in the wells. Furthermore, duplicates of THFM and PEM diluted into dimethyl sulphoxide (DMSO) were tested. After overnight incubation at 37 °C, zones of inhibition around each well were measured using digital callipers (Mitutoyo, Hampshire, UK). The mean inhibition zones of the drug standards were plotted as a regression curve, which was used as a standard graph (Law et al., 1994). This was used to calculate the drug concentrations in the test samples and in the internal controls. To pass the quality control requirement the internal controls had to be within 20 % of the expected concentration of each drug (Law et al., 1994).
The bioassay was validated for linearity, precision, accuracy and reproducibility (FDA, 2001). The limit of detection was calculated as the smallest consistently detectable zone of inhibition (Lower Limit Of Quantification: LLOQ) and an overall coefficient of variation (CV) less than 20 % was accepted. Standard curves were judged linear if the coefficient of determination ($r^2$) using linear regression was $>0.99$. Acceptance criteria dictated that the mean result must be within 20 % of the theoretical concentration of LLOQ, and within 15 % for all other samples (FDA, 2001). The reproducibility of the method was assessed by re-analysing 30 % of the samples of each group on different days and applying Bland-Altman statistical analysis (Altman and Bland, 1983).

**MIC Determination**

Susceptibility testing against chlorhexidine and fluconazole was performed on a total 46 Candida isolates comprising of 32 C. albicans, 13 C. glabrata and 1 C. kefyr isolate. MICs were determined using FLUp and CHX and CLSI M27-A3 microdilution methodology (Clinical Laboratory Standard Institute, 2008a). Briefly, 2-fold dilution series of fluconazole (0.125-2048 mg/L) and chlorhexidine (0.1-50 mg/L) prepared in sterile distilled water and an inoculum of $1 \times 10^3$ cells/ml was used. After 48 h incubation at 37 °C, the growth in each well was measured by spectrophotometry (at 490 nm). For fluconazole, the minimum inhibitory concentration (MIC) was the lowest drug concentration that reduced the OD$_{490}$ by 50 % compared to the drug-free control. The CLSI standard breakpoints for fluconazole were used for susceptibility interpretation (Clinical Laboratory Standard Institute, 2008b). For chlorhexidine, the MIC was the lowest drug concentration that prevented any discernible growth (80 %). Geometric means (GM) and ranges were calculated.
Statistical Analyses

Normality was established allowing a one-way ANOVA (SPSS) to be used to analyse all data and the significance level was set at $P \leq 0.001$. All results were tested using Levene's test for homogeneity of variance ($P < 0.05$), following the assumption of equal variances. Equal variances were confirmed ($P > 0.05$), hence the Bonferroni post hoc test was used to determine the differences in the leached amounts of the impregnated drugs at each time interval.
5.4 Results

A total of 53.0 % of CHX, 38.5 % of FLUc and 13.2 % of FLUp impregnated into the PEM/THFM discs was leached during the 28-day incubation as detected by bioassay (Table 5.1). For all drugs a high rate of initial leaching was followed by a decreased but controlled sustained release during the entire test period of 28 days. Of the total amount leached, 71.8 % of CHX, 75.1 % of FLUc and 70.5 % of FLUp was released during the first week of incubation (Figure 5.1). During the fourth week of incubation 190 mg/L of CHX, 120 mg/L of FLUc and 50 mg/L FLUp was released from the discs (Figure 5.2). The release rate of CHX was significantly higher compared to FLUp and FLUc at all time intervals ($P \leq 0.001$). In addition, a significantly higher amount of fluconazole was leached from the FLUc discs than from the FLUp discs at all time intervals ($P \leq 0.001$). All internal controls were within 10 % of the expected values.
Table 5.1  The cumulative amount (mean ± SD; in mg) of pure fluconazole (FLUp), fluconazole from capsules (FLUc) or chlorhexidine (CHX) released into the distilled water from the acrylic discs during 28-day incubation measured at 6 different time points. Differences between groups were statistically significant at all time points (P≤0.001)

<table>
<thead>
<tr>
<th>days</th>
<th>CHX</th>
<th>FLUc</th>
<th>FLUp</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21.4 (1.5)</td>
<td>17.1 (0.7)</td>
<td>3.6 (0.1)</td>
</tr>
<tr>
<td>1-3</td>
<td>30.8 (1.1)</td>
<td>24.1 (0.1)</td>
<td>6.8 (0.1)</td>
</tr>
<tr>
<td>3-7</td>
<td>38.1 (0.2)</td>
<td>28.9 (0.2)</td>
<td>9.3 (0.1)</td>
</tr>
<tr>
<td>7-14</td>
<td>44.4 (0.2)</td>
<td>32.6 (0.1)</td>
<td>10.9 (0.1)</td>
</tr>
<tr>
<td>14-21</td>
<td>49.2 (0.1)</td>
<td>36.1 (0.1)</td>
<td>12.2 (0.0)</td>
</tr>
<tr>
<td>21-28</td>
<td>53.0 (0.1)</td>
<td>38.5 (0.2)</td>
<td>13.2 (0.0)</td>
</tr>
</tbody>
</table>

Figure 5.1  Proportion of drug leached from discs impregnated with chlorhexidine (CHX), fluconazole from capsules (FLUc) and pure fluconazole (FLUp) during the 28-day incubation. Cumulative percentage presented
Figure 5.2 MICs (mg/L) of 46 isolates (left y-axis) and the amount drugs released from discs impregnated with fluconazole powder from capsules (a) pure fluconazole (b) and chlorhexidine (c) during the 28-day incubation (right y-axis)
Antifungal activity was confirmed for CHX, FLUc and FLUp impregnated disc leachates by clear inhibition zones of candidal growth compared to the known drug standards in the bioassay (Figure 5.3). The leachates of the negative control discs (acrylic without drug) or the two components of the polymer alone (THFM and PEM) showed no inhibition of candidal growth. Bland-Altman analyses for the repeated measurements (9 in each group) showed minimal variation between results and high reproducibility (Figure 5.4). The assay was linear with \( r^2 > 0.99 \) and the limits of detection were 6.25 and 50 mg/L for fluconazole and chlorhexidine, respectively. Accuracies were within 7 % for fluconazole and 8 % for chlorhexidine, and variability (precision) was <3 % for both fluconazole and chlorhexidine.

Figure 5.3 A photograph of a representative bioassay plate showing growth inhibition zones formed by diluted leachates of chlorhexidine (a) and fluconazole (FLUc) (b) discs in distilled water. A random template was used for well selection. Positive controls in 1A wells, negative controls e.g. in 2C (b) and 1D (b), THFM controls in 4E (b) and 3B (b) and PEM controls in 6B (b) and 6E (b)
Figure 5.4 The reproducibility of the bioassay was assessed by re-analysing 30% of the samples of each group (CHX, FLUc, FLUp). A Bland-Altman plot of 27 repeated measurements (9 each group) shows minimal variation between results and high reproducibility. Each dot represents the mean concentration of two measurements against the difference between the two measurements. 95% Confidence Intervals for the first measurements were 4.93-10.84 and were 4.69-10.24 for the repeated measurements.

The MIC values of fluconazole and chlorhexidine against 46 isolates of *Candida* are shown in Figure 5.2. The GM MIC of all isolates was 1.83 mg/L (0.25-256) for fluconazole and 4.77 mg/L (0.78-6.25) for chlorhexidine. The GM MIC of the *C. albicans* isolates for fluconazole was 0.61 mg/L (0.25-256) and for chlorhexidine 5.03 mg/L (1.56-6.25) while the GM MIC of the *C. glabrata* isolates for fluconazole was 30.34 mg/L (8-128) and for chlorhexidine 4.78 mg/L (3.125-6.25). The MIC for the one *C. kefyr* isolate for fluconazole was 0.5 mg/L and for chlorhexidine was 0.78 mg/L.
5.5 Discussion

The present study shows that it is feasible to employ the PEM/THFM polymeric system for sustained release of antifungal agents up to 28 days and accordingly our hypothesis has been accepted. Furthermore, our results clearly indicate that both chlorhexidine and fluconazole are readily leached from the polymer and polymerisation of the acrylic does not affect the antimicrobial activity of the incorporated agents even in prolonged incubation. Importantly, the amount of drug released from the discs each week exceeded the MICs of most isolates. The amount of chlorhexidine released from the discs during the last week of incubation exceeded the MICs of all isolates, and the amount of fluconazole released exceeded the MICs of 97.8 % to 91.3 % of the isolates during the second week of incubation and 91.3 % to 89.13 % of the isolates during the last week of incubation for FLUc and FLUp, respectively. Following the initial high release, the elution of drugs showed a slow and steady diffusion without reaching a plateau after 28 days. The slower diffusion is likely to be attributed to the diffusion of the drug from the core of the polymer by water cluster formation around the drug particles controlled by concentration dependent diffusion (Patel et al., 2001; Darwish et al., 2011). The polymeric system used in this study is characterised by distinctive water absorbing capability and sustained drug release (Sawtell et al., 1997).

The amount of chlorhexidine released was significantly higher than that of the two fluconazole preparations. This is in agreement with previous findings using a different polymer carrier (Amin et al., 2009). However, the amount of chlorhexidine released in our study was markedly higher than previously reported for PEM/THFM carrier using spectrophotometry (Patel et al., 2001). This may be due to the differences in the dimensions and surface areas of the discs. A proportionally larger surface area exposes
more drug particles to the soaking water and thus enhances drug release (Patel et al., 2001; Amin et al., 2009). Moreover, spectrophotometry is generally regarded to be less sensitive than bioassay as a detection method, although there is no single study to confirm this. Significant differences were detected in the leaching of fluconazole from the discs impregnated with pure fluconazole and fluconazole powder from capsules. This is likely to be due to the presence of impurities in the powder from capsules and the need to replace a higher proportion of the PEM powder in order to incorporate equivalent amount of active drug into the discs. This may disrupt the polymer structure and allow the drug to leach more easily. The formulation of fluconazole used in previous studies was not clearly defined whereby both formulations were included in our study. Fluconazole capsules are readily available to dental practitioners and based on the results of this study could be useful in clinical practice.

Highly inhibitory concentrations of chlorhexidine and fluconazole released from the PEM/THFM discs. The concentrations of fluconazole released from FLUc discs ranged from 2380 to 197 times higher than GM MIC for C. albicans, 47 to 4 times for C. glabrata, and 2890 to 240 times for C. kefyr during the first and last weeks, respectively. One C. albicans and three C. glabrata isolates with high MIC values for fluconazole and designated as resistant were included in order to test the efficacy of the PEM/THFM on difficult to treat organisms. The concentrations of fluconazole released from discs impregnated with FLUc were higher than the MICs of the resistant C. glabrata isolates up to 21 days of incubation but only up to 3 days of leaching of FLUp discs. The MIC for the resistant C. albicans was higher than the amount of fluconazole released from FLUc discs after 3 days of incubation and at all times of that of pure fluconazole.
The released concentrations of chlorhexidine ranged from 379 to 38 times higher than the GM MIC for *C. albicans*, 399 to 40 times than the GM MIC for *C. glabrata* and 2442 to 243 times than the GM MIC for *C. kefyr* during the first and last week respectively. No cross-resistance between fluconazole and chlorhexidine was detected and the leached concentrations of chlorhexidine were higher than the MICs of all isolates for it at all time points. The components of the polymer system showed no antimicrobial effect.

Direct comparison of our results with previously reported studies is difficult due to differences in the methodologies, drug concentrations, formulations and detection methods used. However, the results of the present study are in line with those of Ryalat *et al.* (2011) showing similar release and efficacy of chlorhexidine from PEM as they did from PMMA. Truhlar *et al.* (1994) have demonstrated a similar decrease of antifungal activity during extended water immersion but in their study the released concentrations were not compared with MICs of candidal isolates as in the present study. The main limitation of the present and the above-mentioned *in vitro* studies is that further *in vivo* work is warranted to determine their clinical relevance. Nevertheless, the quantitative and qualitative bioassay method used in the present study could be a valuable tool for evaluation of other polymeric systems and other antifungal agents both *in vitro* and *in vivo*. Whilst HPLC may be a more accurate detection method (Amin *et al.*, 2009; Ryalat *et al.*, 2011), the bioassay method demonstrated good sensitivity and reproducibility (Andes *et al.*, 2009).
5.6 Conclusions

The PEM/THFM polymeric system was found to be an effective carrier for sustained release of antifungal agents up to 28 days. However, the polymeric system impregnated with CHX or FLU showed remarkable antifungal activity against a wide range of *Candida* isolates and the released concentrations were within the non-toxic range (Rushton, 1977). The choice of the antifungal agent depends mainly on the antimicrobial profile of the causative agent, chronicity of the predisposing factors, immunological status of the patient and the presence of biofilms. Chlorhexidine could be used effectively when an azole has failed, in immunocompromised patients with a need for long-term treatment, as well as when mixed bacterial and fungal biofilms are suspected. On the other hand, fluconazole is well tolerated with few adverse effects and can be successfully used to treat uncomplicated infections in otherwise healthy patients where risk for development of resistance is limited. Therefore, a polymeric system containing CHX or FLU could be a very promising treatment modality as the drug is effective and directed to the site of pathology.
5.7 Acknowledgements

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CHAPTER 6

Candidacidal effect of fluconazole and chlorhexidine released from acrylic polymer

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6.1 Abstract

Objectives: To investigate the efficacy and rate of killing of a fluconazole or chlorhexidine impregnated polymeric delivery systems against fluconazole-susceptible and -resistant Candida albicans and fluconazole-resistant C. glabrata.

Methods: Poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) discs impregnated with chlorhexidine (CHX), pure fluconazole (FLUp), or fluconazole from capsules (FLUc) were prepared by substituting a portion of the PEM powder with an equivalent amount of each drug. Discs were incubated in sterile water for 1, 3, 7, 14, 21 and 28 days. The amounts of drugs in the leachates were measured spectrophotometrically and their antifungal activity against fluconazole-susceptible and -resistant candidal isolates was determined using a time-kill method and by comparing the released concentrations to the corresponding MICs.

Results: Fluconazole and chlorhexidine were leached from PEM/THFM polymer for up to 28 days and the released concentrations were fungicidal against the three Candida isolates for at least the first 7 days. CHX leachates killed all Candida isolates more rapidly than the two fluconazole formulation leachates throughout the study period. FLUc leachates required longer incubation for 100 % killing than FLUp leachates. The proportion of viable C. glabrata dropped more slowly than that of C. albicans with the same MIC.

Conclusions: The concentrations of chlorhexidine and fluconazole leached from PEM/THFM polymer were fungicidal against all Candida isolates, including those resistant to fluconazole, for the first 7 days. Chlorhexidine leachates showed a rapid
fungicidal activity for up to 4 weeks which can be of use in cases with poor response to conventional antifungals.

**Key words:** fluconazole, chlorhexidine, *Candida*, leachates, biomaterial infection.
6.2 Introduction

Oral candidosis is a prevalent problem amongst the elderly and medically compromised patients (Figueiral et al., 2007; Coco et al., 2008). It is a complex inflammatory process involving formation of candidal-bacterial biofilms on the various natural and artificial surfaces present in the oral cavity. Candida species are common oral colonisers and they readily form mixed species biofilms on non-renewing surfaces such as teeth, dental fillings, intubation tubes and dentures (Ramage et al., 2006). These biofilms form a potential source of systemic infection especially as they are inherently resistant to antimicrobial treatment (Rautemaa and Ramage, 2011). They are also associated with a high level of mucosal inflammation locally (Ramage et al., 2006; Coco et al., 2008).

Peroral fluconazole is commonly used for treatment as it is well tolerated with few side effects (Rautemaa and Ramage, 2011; Versalovic, 2011). However, microbiological and clinical resistance is a major clinical concern. This has been associated with selection and emergence of fluconazole-resistant Candida species such as C. glabrata as well as poor efficacy against candidal-bacterial biofilms (Ramage et al., 2002a; Ramage et al., 2011). Moreover, in patients with reduced saliva production therapeutic drug levels are difficult to achieve in the oral cavity with systemic therapy (Siikala et al., 2010). Interestingly, alternative topical agents such as chlorhexidine have proven exceptional efficiency against Candida species (Meiller et al., 2001; Redding et al., 2009). In addition, chlorhexidine has a unique anti-biofilm activity with low MIC values (Lamfon et al., 2004; Redding et al., 2009).
One of the main challenges in topical treatment is the rapid clearance of an administered drug from the site of infection by saliva (Samaranayake et al., 2009). Moreover, rigid patient compliance for frequent drug administration is paramount for an optimal outcome (Samaranayake et al., 2009). Therefore, alternative approaches are needed. Local self-release delivery systems have been suggested as an alternative approach for achieving and maintaining therapeutic drug levels at the site of infection (Douglas, 1977; Patel et al., 2001; Amin et al., 2009; Darwish et al., 2011). Schneid and colleagues (1992) showed that leachates of soft acrylic lining material discs impregnated with chlorhexidine, fluconazole, nystatin or clotrimazole inhibited candidal growth. It has also been demonstrated that chlorhexidine and fluconazole become released steadily in anti-candidal concentrations from a hard poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate acrylic (PEM/THFM) for up to 4 weeks (Salim et al., 2012a). However, the fungicidal potential and the rate of killing of such leachates have not been studied previously.

The aim of the present study was to investigate the efficacy of fluconazole or chlorhexidine impregnated PEM/THFM delivery systems against fluconazole-susceptible and -resistant C. albicans and fluconazole-resistant C. glabrata using a time-kill approach. A further aim was to compare the rate of killing of the two agents and the impact of the fluconazole formulation used on this.
6.3 Materials and methods

Study Design

Acrylic discs were prepared using (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) impregnated with chlorhexidine (CHX), pure fluconazole (FLUp), or fluconazole from capsules (FLUc) by substituting a portion of the PEM powder by an equivalent amount of each drug. The drug-free control discs were prepared following manufacturer's instructions. All discs were incubated in sterile distilled water at 37 °C for 28 days. The leachates were collected and the water was replaced at 1, 3, 7, 14, 21 and 28 days. The amounts of released drugs were measured using a spectrophotometer and the antifungal activity of the leachates was determined using a time-kill method. The MIC of the Candida isolates for each drug was determined and compared to the released concentrations. The experiment was performed in quintuplicate.

Preparation of PEM/THFM Discs

For the control discs, 1 g of PEM (Lucite International, Durham, UK) and 0.6 ml of THFM (Sigma-Aldrich, Dorset, UK) were mixed. For the impregnated discs, 10 % (w/w) of the PEM was replaced with CHX (Sigma-Aldrich), 10 % (w/w) with FLUp (Pfizer, Kent, UK) or 25 % (w/w) with FLUc (Pfizer) resulting in 100 mg of active drug per disc, for all. The drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed. All mixtures were packed into disc shaped steel moulds (40 mm diameter and 0.5 mm height) and allowed to cure for 15 min.
Disc Incubation

Each disc was soaked individually in 20 ml of sterile distilled water at 37 °C in tightly sealed plastic flasks. The flasks were gently shaken by hand three times daily throughout the experiment to simulate the oral environment. The leachates of the drug-free control discs were used as negative controls.

Drug Concentration Measurement

The released amount of drugs was quantified using a UV spectrophotometer at 220 nm (Shimadzu, Kyoto, Japan) (Wilson and Wilson, 1993). Triplicate 10-fold dilution series of the leachates were analysed at 1, 3, 7, 14, 21 and 28 days. The absorbance readings of the drug-free control leachates were used as a reference. The unknown concentrations of drug in each leachate were calculated using standard curves of known concentrations of chlorhexidine or fluconazole.

Isolates and Inoculum Preparation

One Candida strain susceptible to fluconazole and two isolates resistant to fluconazole were used: C. albicans ATCC 90028 (MIC of 0.25-1 mg/L), and C. albicans F/2511 and C. glabrata F/4023 (reported with MIC >64 mg/L, for both). The two latter isolates were obtained from the culture collection of the Mycology Reference Centre (Manchester, UK). Isolates were grown on Sabouraud agar for 48 h and then a couple of colonies were inoculated into 10 ml of distilled water and the cell density was adjusted spectrophotometrically (BMG Labtech, Aylesbury, UK) to $1.0 \times 10^6$ cells/ml and further diluted to $1.0 \times 10^5$ cells/ml final cell density (working dilution).
**MIC Determination**

MICs were determined for FLUc, FLUp and CHX and using CLSI M27-A3 microdilution methodology (Clinical Laboratory Standard Institute, 2008a). Briefly, 2-fold dilution series of FLUp and FLUc (0.125-2048 mg/L) and CHX (0.1-50 mg/L) were prepared in sterile distilled water and an inoculum of $1 \times 10^3$ cells/ml was used. After 48 h incubation at 37 °C, the growth in each well was measured by spectrophotometry (BMG Labtech) at 490 nm. For fluconazole, the MIC was the lowest drug concentration that reduced the OD$_{490}$ by 50 % compared to the drug-free control. The CLSI standard breakpoints for fluconazole were used for susceptibility interpretation (Clinical Laboratory Standard Institute, 2008b). For chlorhexidine, the MIC was the lowest drug concentration that reduced the OD$_{490}$ by 80 % compared to the drug-free control (Clinical Laboratory Standard Institute, 2008b).

**Time-kill Studies**

Time-kill studies were carried out following standard protocols (Klepser et al., 1998). To detect the fungicidal activity of the leachates, 100 µl of the working dilution was transferred to sterile tubes containing 900 µl of leachate of CHX, FLUp, FLUc and the drug-free control discs. The tubes were incubated at 37 °C and aliquots were removed at 1, 2, 4, 6, and 24 h post inoculation. Ten microlitre aliquots of the neat sample and two 10-fold serial dilutions were plated on Sabouraud agar (Oxoid, Basingstoke, UK) and were incubated for 48 h at 37 °C to test for viability. This was repeated for all leachates collected at 3, 7, 14, 21, 28 days and against the three isolates tested. Viability counts of the working dilutions were used as controls. A fungicidal effect was defined as 100 % kill. Three hundred data points were collected.
for each of the six time intervals of leaching: 4 disc types, 5 replicates, 5 time-kill measurements and 3 isolates.

*Statistical Analyses*

One-way ANOVA was used to analyse all data and the significance level was set at $P \leq 0.05$. All results were tested using Levene’s test for homogeneity of variance ($P \leq 0.05$), following the assumption of equal variances. Equal variances were confirmed ($P > 0.05$), hence the Bonferroni *post hoc* test was used to determine the differences in the leached amounts of the impregnated drugs at each time interval.
6.4 Results

The impregnated drugs were released from the PEM/THFM discs over the 28-day period with a high rate of initial leaching followed by controlled slow release (see key in Figures 6.1, 6.2, 6.3). At all time points, the rate of release of CHX and FLUc was significantly higher compared to FLUp (P≤0.05). The MIC for CHX of all isolates was 6.25 mg/L. The MIC for FLUp and FLUc of C. albicans ATCC 90028 was 0.25 mg/L and was 128 mg/L for both C. albicans F/2511 and C. glabrata F/4023.

For CHX and FLUp, all leachates with concentrations above the MIC (range 173-1019 mg/L and 68-195 mg/L, respectively), led to 100 % killing of all three isolates during 24 h incubation. No significant killing was detected for leachates with concentrations below the MIC (Figures 6.1 and 6.2). All FLUc leachates (concentration range 116-834 mg/L) resulted in 100 % killing of the fluconazole-susceptible isolate. Of the two fluconazole-resistant isolates, 100 % killing was detected with leachates collected during the first 7 days (concentration range 262-834 mg/L; Figure 6.3). Over 2 × MIC was required for 100 % killing with the FLUc compared to 1.2 × MIC with FLUp. A concentration of 1.95 × MIC of FLUc resulted in 60 % killing of the resistant isolates in 24 h incubation.

Leachates from CHX impregnated discs collected at all time intervals resulted in 100 % killing of all three Candida isolates during 60 min incubation (Figure 6.1). All FLUp leachates led to 100 % killing of the fluconazole-susceptible C. albicans ATCC 90028 within 60 min (Figure 6.2a). For the fluconazole-resistant C. albicans F/2511 100 % killing was achieved within 60 min with FLUp leachates collected during the first 7 days (Figure 6.2b). For C. glabrata F/4023 100 % killing was seen at 2 h, 4 h
and 24 h time points with leachates collected on days 1, 3 and 7, respectively (Figure 6.2c). The killing efficiency of leachates collected after 7 days was insignificant against both *C. albicans* F/2511 and *C. glabrata* F/4023 (Figures 6.2b and c).

**Figure 6.1** Representative time-kill curve plots for *C. albicans* ATCC 90028 (a), *C. albicans* F/2511 (b), and *C. glabrata* F/4023 (c) of leachates of different time intervals of chlorhexidine impregnated discs. The MIC of all isolates was 6.25 mg/L for chlorhexidine.
Figure 6.2 Representative time-kill curve plots for *C. albicans* 90028 (a), *C. albicans* F/2511 (b), and *C. glabrata* F/4023 (c) of leachates of different time intervals of pure fluconazole impregnated discs. The MIC of *C. albicans* 90028 for pure fluconazole was 0.25 mg/L while the MIC of *C. albicans* F/2511 and *C. glabrata* F/4023 was 128 mg/L.

All FLUc leachates led to 100 % killing of *C. albicans* ATCC 90028 within 24 h incubation (Figure 6.3a). For *C. albicans* F/2511 100 % killing was seen in 2 h incubation with leachates collected on day 1 and in 24 h incubation time points with leachates collected on days 3 to 7 (Figure 6.3b). For *C. glabrata*, leachates collected
during the first 7 days resulted in 100 % of killing during 24 h incubation. FLUc leachates collected between days 7-14 resulted in 60 % killing during 24 h incubation compared to 20 % for equivalent FLUp leachates of both \textit{C. albicans} F/2511 and \textit{C. glabrata} F/4023 (Figure 6.3b and c).

Figure 6.3 Representative time-kill curve plots for \textit{C. albicans} ATCC 90028 (a), \textit{C. albicans} F/2511 (b), and \textit{C. glabrata} F/4023 (c) of leachates of different time intervals of fluconazole from capsules impregnated discs. The MIC of \textit{C. albicans} ATCC 90028 for fluconazole from capsules was 0.25 mg/L while the MIC of \textit{C. albicans} F/2511 and \textit{C. glabrata} F/4023 was 128 mg/L.
6.5 Discussion

The present study shows that fluconazole and chlorhexidine become readily leached from the PEM/THFM polymeric system for up to 28 days and that the released concentrations were fungicidal against the three *Candida* isolates, including the two isolates resistant to fluconazole, for at least the first 7 days. Significant differences between the rates of killing between the released agents were detected. CHX leachates killed all *Candida* isolates more rapidly than either of the two fluconazole formulation leachates. Interestingly, FLUc leachates required longer incubation for 100 % killing than FLUp leachates although higher concentrations of active drug were measured in the leachates at all time points and the MIC of both fluconazole compounds was the same for the isolates. In addition, marked differences in the rate of killing between the two fluconazole-resistant isolates were detected with both fluconazole formulations: the proportion of viable *C. glabrata* F/4023 dropped more slowly than that of the *C. albicans* F/2511 with the same MIC. A PEM/THFM polymer impregnated with either fluconazole or chlorhexidine has previously been shown to have antifungal activity but the rate of killing or the impact of the formulation used on this has not been studied before (Patel *et al.*, 2001; Salim *et al.*, 2012a).

All CHX leachates were equally effective and rapid in killing fluconazole-resistant and susceptible isolates for up to 28 days. This can be explained by the high concentrations of CHX released exceeding the MIC values (6.25 mg/L) for all isolates at all time intervals. This can also be further explained by the rapid uptake of CHX by *C. albicans* and *C. glabrata* which has been reported previously (Hiom *et al.*, 1995).

The present work extends the findings of previous studies where a similar polymeric system impregnated with CHX or FLU was used and efficacy against
fluconazole-resistant *C. albicans* was seen (Amin *et al.*, 2009; Ryalat *et al.*, 2011). However, in these previous studies the kinetics of killing and the impact of different fluconazole formulations were not defined. The present study shows that CHX leachates killed all *Candida* isolates in one hour compared to the two fluconazole formulation leachates where up to 24 h was required.

Significantly higher concentrations of fluconazole were detected in leachates from discs impregnated with fluconazole powder from capsules than those impregnated with pure fluconazole as reported before using a different detection method (Salim *et al.*, 2012a). However, day 1 leachates of FLUc required 2-24 h for 100 % killing in contrast to 1-2 h for the FLUp leachates despite over four times higher concentration of active drug being leached FLUc discs compared to FLUp discs. Both of the fluconazole-resistant isolates were killed with the FLUp leachates when the concentration was higher than the MIC but 2 × MIC was required for this with the FLUc leachates. When killing of the fluconazole-resistant isolates was seen it was slower than that of the susceptible isolate. In addition, the killing of *C. glabrata* was slower than that of the fluconazole-resistant *C. albicans* which is in agreement with previous reports (Li *et al.*, 2003). These unexpected differences between the two formulations may be due to the excipients in FLUc which may interfere with drug uptake by *Candida* and modify the action of the drug. Slow rate killing has significant consequences as it may lead to selection and emergence of resistance (Rautemaa and Ramage, 2011).

The findings of the current study clearly demonstrate that both chlorhexidine and fluconazole are readily leached from the polymer and the antifungal activity of the impregnated agents has not been affected by acrylic polymerisation even after prolonged incubation. A time-kill approach was used in this study to show precisely
how quickly the agents will act against the tested isolates which was a limitation of previously used methods such as CFU count and well diffusion test (Patel et al., 2001; Darwish et al., 2011). Although a limited number of isolates were studied, the two species included in this study, *C. albicans* and *C. glabrata*, represent those most commonly associated with denture-induced stomatitis (Coco et al., 2008). Based on our findings the released amounts of the antimicrobial agents were the highest during the first day (20 mg of CHX, 17 mg FLUc). For an average full denture this would be equivalent to 37 mg of CHX and 30 mg of FLU (Poštić, 2011). These amounts are safe and within the recommended doses (Rushton, 1977; Force and Nahata, 1995). As this is an *in vitro* study, further clinical studies are warranted to determine its clinical applicability.
6.6 Conclusions

A polymer containing either chlorhexidine or fluconazole is a promising treatment modality and effective amounts of drugs could be released at the site of pathology. The rapid kill rate of chlorhexidine compared with fluconazole is encouraging and may prove to be an effective option for example when azole antifungal treatment fails, in immunocompromised patients with a need for long-term treatment or when fungal biofilms are suspected. Moreover, the common side effects experienced with chlorhexidine mouth rinse including superficial staining of teeth and oral mucosa and gustatory dysfunction can be minimised by the targeted release of the delivery device. On the other hand, the well tolerated fluconazole appears to have potential for treatment of uncomplicated infections in otherwise healthy patients where risk for development of resistance is limited.
CHAPTER 7

Chlorhexidine impregnated PEM/THFM polymer exhibits superior activity to fluconazole against *Candida albicans* biofilm formation

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7.1 Abstract

Biofilm-associated infections represent a major challenge for biomaterials. Methods to alter their chemical characteristics offer an attractive solution for enhanced microbial control. The aim of this study was to investigate the efficacy of a poly (ethyl methacrylate)/tetrahyrofurfuryl methacrylate (PEM/THFM) acrylic model impregnated with fluconazole (FLU) or chlorhexidine (CHX) in preventing Candida biofilm formation in vitro. PEM/THFM discs impregnated with CHX (n=50), FLU (n=50) and drug-free control discs (n=50) were infected with Candida albicans ATCC 90028. Discs were incubated for 2, 7, 14, 21 or 28 days at 37 °C, and the biofilm biomass and metabolic activity was quantified at each time point. FLU discs were shown to exhibit poor overall biofilm inhibition characteristics, with a mean metabolic and biomass inhibition of 12.6 % and 8.8 %, respectively. Conversely, CHX discs were highly effective, significantly inhibiting biofilm development by 75 % (P≤0.001) and its metabolism by 84 % (P≤0.001) for all time points tested. The notable efficacy of CHX against C. albicans biofilms is a promising outcome to overcome the side effects and poor relative activity of conventional antifungal agents against Candida biofilms. These findings indicate that impregnation of PEM/THFM with antimicrobials has potential as a treatment modality for denture-induced stomatitis.

Key words: poly (ethyl methacrylate)/tetrahyrofurfuryl methacrylate, antimicrobial, Candida Biofilm, biomaterial infection, bone cement.
7.2 Introduction

The majority of human microbial infections involve microbial biofilms, complex communities of microorganisms encased within an extrapolymeric matrix material (Costerton et al., 1999; Potera, 1999). These protected communities have unique characteristics that confer their survival and pathogenicity compared to a planktonic lifestyle (Ramage et al., 2005). The most important genus of fungal pathogens to humans, Candida, is known for its ability to form biofilms (Ramage et al., 2005; Rautemaa and Ramage, 2011), of which C. albicans predominates (Coco et al., 2008).

Candida spp. have the ability to form biofilms on various types of medical devices, including catheters, stents, shunts, prostheses, implants, as well as the dentition and other non-shedding artificial or natural surfaces (Donlan, 2001). Resistance of Candida biofilms to antimicrobial agents is a serious clinical problem and of major concern (Ramage et al., 2005). Whilst fluconazole is commonly used for the treatment of candidal infections (Rautemaa and Ramage, 2011), its efficacy on biofilms is relatively poor (Ramage et al., 2002a; Ramage et al., 2011). Whereas chlorhexidine, a commonly used topical disinfectant, has antimicrobial activity against a wide range of microorganisms including Candida (Giuliana et al., 1999; Meiller et al., 2001). In addition, it has been found to have superior activity against candidal biofilms compared to fluconazole (Lamfon et al., 2004; Ramage et al., 2011).

Antimicrobial impregnation of medical devices has been suggested to have potential for the prevention of microbial adherence, the first step of biofilm formation (Cho et al., 2001; Samuel and Guggenbichler, 2004). Slow release of an antimicrobial from a material also potentially inhibits biofilm maturation (Cho et al., 2001). Incorporation of antibiotics into cements used in orthopaedic surgery is common.
practice (Frutos et al., 2010). The use of such drug delivery systems allows continuous drug release to the site of infection with minimal risk of sub-therapeutic levels or systemic toxicity (Patel et al., 2001; Amin et al., 2009; Salim et al., 2012a). Moreover, the use of self-releasing systems requires minimal intervention and monitoring (Brook et al., 1986). Such systems have been shown to be highly effective against planktonic candidal cells in vitro (Addy, 1981; Patel et al., 2001; Geerts et al., 2008; Amin et al., 2009). It has been shown previously that chlorhexidine and fluconazole incorporated into self-cured poly (ethyl methacrylate) and tetrahydrofurfuryl methacrylate (PEM/THFM) polymeric system become readily leached from the polymer in a microbiologically active form for up to 4 weeks (Salim et al., 2012a). This system could potentially be used as a denture liner for the treatment of denture-induced stomatitis, a difficult-to-treat candidal-bacterial biofilm infection with high relapse rate (Ramage et al., 2004; Coco et al., 2008).

Self-curing systems based on PEM/THFM have been suggested to have potential for various prosthetic applications. PEM/THFM has a number of superior characteristics compared with methyl methacrylate and n-butyl methacrylate based systems. PEM/THFM has a low exothermic reaction compared to methyl methacrylate and superior biological properties compared to n-butyl methacrylate based systems (Patel and Braden, 1991a; Patel et al., 1994). It has also been shown to support the growth of chondrocytes whilst being non-irritant in dental use (Pearson et al., 1986; Wyre and Downes, 2000). Moreover, it has been documented to be a ductile material contributing to its physical biocompatibility (Patel and Braden, 1991c).

Various biofilm model systems have been used to study candidal biofilm formation and susceptibility of these preformed biofilms to antifungal agents.
(Lamfon et al., 2004; Ramage et al., 2005). However, the ability of acrylic polymers impregnated with antifungal agents to resist *Candida* biofilm formation has not been studied previously. There is a clear clinical need for the evaluation of the impact of impregnation with antifungal agents on *Candida* biofilm formation. An *in vitro* model simulating biofilm formation in the oral cavity was used. The aim of the present study was to investigate the efficacy of a PEM/THFM model system impregnated with either chlorhexidine or fluconazole in preventing *Candida* biofilm formation *in vitro*. The hypothesis was that impregnation of PEM/THFM with chlorhexidine or fluconazole can inhibit *Candida* biofilm formation.
7.3 Materials and methods

Study Design

One hundred discs of poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) impregnated with fluconazole (FLU; n=50) or chlorhexidine (CHX; n=50) were prepared by replacing a portion of the PEM powder by each drug (described in detail below). Drug-free control discs (n=50) were prepared following manufacturer's instructions. All discs were placed into 24-well microtitre plates and exposed to *C. albicans*. Plates were incubated for 2, 7, 14, 21 or 28 days at 37 °C. The metabolic activity of the biofilm was quantified using XTT metabolic dye assay and the biofilm biomass was quantified using crystal violet staining (CV). At each time point, ten replicate discs impregnated with CHX, ten replicate discs impregnated with FLU and ten drug-free control discs were used for XTT and CV analyses. The inhibition of biomass formation and metabolic activity was used to assess the efficacy of the impregnated PEM/THFM model system.

Preparation of PEM/THFM Discs

To prepare the control discs, 5 g of PEM acrylic resin (Lucite International, Durham, UK) and 3 ml of THFM monomer (Sigma-Aldrich, Dorset, UK) were mixed and packed into a disc shaped mould (10 mm diameter and 1.0 mm height) and allowed to cure under 2 bar pressure for 15 min. To prepare the impregnated discs, 10 % (w/w) of the PEM powder was replaced with fluconazole (Pfizer, Kent, UK) or chlorhexidine powder (Sigma-Aldrich) (resulting in 12.5 mg of active drug per disc, for all). The drugs were first mixed into the PEM powder, then poured into the THFM liquid monomer and mixed. The discs were prepared as described above.
**Inoculum Preparation**

*C. albicans* ATCC 90028 was freshly grown on Sabouraud agar (Oxoid, Basingstoke, UK) and incubated for 48 h prior to inoculation into 20 ml of yeast peptone dextrose (YPD) medium (1 % w/v yeast extract, 2 % w/v peptone, 2 % w/v dextrose; Oxoid). This was incubated in an orbital shaker (100 rpm) overnight at 37 °C to produce budding yeasts. After incubation, the cells were harvested and washed in sterile phosphate buffered saline (PBS). Then the cells were resuspended into RPMI-1640 (Sigma-Aldrich) supplemented with L-glutamine and buffered with morpholinepropanesulfonic acid (MOPS; Oxoid). The cell density was adjusted to 1.0 × 10^6 cells/ml to produce the biofilm inoculum (Ramage *et al.*, 2001). The planktonic MIC (0.25 mg/L) for fluconazole for the isolate was confirmed following the CLSI method.

**Biofilm Formation**

All discs were rinsed with sterile water three times and then soaked in PBS for 5 min and then rinsed three times with sterile water and placed into 24-well plates. Two millilitres of the *C. albicans* inoculum in RPMI medium was added into each well (2.0 × 10^6 cells/disc), and the plates were incubated at 37 °C. The RPMI-1640 medium was removed and replaced at 2, 7, 14, 21 and 28-day time points, and at the same time points both the biofilm biomass and metabolism quantified.

**XTT Reduction Assay**

A semi-quantitative measure of each biofilm was calculated using XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide) reduction assay (Ramage *et al.*, 2001). Before analyses the discs were transferred to a sterile 24-well plate and washed three times with sterile PBS. In brief, XTT (Sigma-Aldrich) was
dissolved in PBS to produce a saturated solution at 0.5 g/L. This solution was filtered, aliquoted and stored at -80 °C. Before each assay, an aliquot was thawed and menadione (10 mM prepared in acetone; Sigma-Aldrich) was added to a final concentration of 1 µM. A 500 µl aliquot of XTT/menadione solution was added to each pre-washed biofilm and to the positive and negative control wells to measure background XTT reduction levels. The plates were covered with aluminium foil and transferred to an incubator at 37 °C for 3 h and then the colorimetric changes were measured using a spectrophotometer (BMG Labtech, Aylesbury, UK) at 490 nm.

Both negative and positive controls were included. Negative controls consisted of discs incubated in RPMI without yeast inoculation (3 replicates for each group CHX, FLUp and the drug-free control), which were used as baseline measurements for their corresponding discs that incubated in RPMI with yeast inoculation. The positive controls were the drug-free acrylic discs with yeast inoculation.

*Biofilm Biomass Quantification*

The biomass of the biofilms was measured using the crystal violet staining method as described by Mowat *et al* (2007). Before analyses the discs were transferred to a sterile 24-well plate and washed three times with sterile PBS. Briefly, the discs were first allowed to air-dry and then 500 µl of 0.5 % (w/v) crystal violet solution (Sigma-Aldrich) was added for 5 min. The discs were then gently washed under running water and treated with 500 µl of 95 % ethanol (Sigma-Aldrich) for 1 min. From each well, 100 µl was transferred to a clean 96-well microtitre plate and the absorbance of the collected solutions was assessed using spectrophotometer (BMG Labtech) at 584 nm.
Statistical Analysis

Data were entered into a statistical software package SPSS 18.0 (SPSS Inc., Chicago, Illinois, USA) and analysed with one-way ANOVA. The Dunnett T3 post hoc test was used to compare the differences in XTT and CV values between the disc types and time points. The significance level was set at $P \leq 0.05$. The correlation between the XTT and CV values was determined using Pearson’s correlation test.
7.4 Results

*C. albicans* biofilm metabolic activity from the drug-free PEM/THFM control discs increased continuously during the 28-day incubation as quantified by XTT (Figure 7.1), with the most rapid increase observed between 2 to 7 days (*P*=0.047). The biofilm biomass also increased continuously during the 28-day incubation (Figure 7.2), increasing most rapidly between 7 and 14 days (*P*=0.04). There was a strong positive correlation between the *C. albicans* biofilm biomass and metabolism on all disc types (*R*=0.955, *P*<0.001).

*C. albicans* biofilms grown on the FLU impregnated discs increased continuously throughout the 28-day incubation. Metabolic activity increased most rapidly between 2 to 7 days (*P*<0.001), and the biomass increased continuously during the 28-day incubation (Figures 7.1 and 7.2). A rapid and significant increase was observed between 2 and 7 days (*P*=0.039) and between 7 and 14 days (*P*=0.009). These discs inhibited biofilm metabolism by mean (12.6 % [range 2-40 %]) compared to the drug-free control discs. Significant metabolic inhibition was only observed at the 2-day time point (*P*=0.004). Biofilm biomass inhibition was only significant at the 2-day time point (*P*=0.047).

Biofilms grown on the CHX impregnated acrylic discs showed no significant increase in metabolism (Figure 7.1) or biomass (Figure 7.2) over the 28 day period, with only a minor increase observed at day 14. Discs impregnated with CHX significantly inhibited *C. albicans* biofilm metabolic activity (84 % [range 80-85 %]) compared to the drug-free control discs (*P*<0.001 for all) and FLU impregnated discs (*P*<0.001 for all) (Figure 7.1). Moreover, these discs inhibited biofilm biomass significantly more
(75 % [range 70-75.5 %]) than FLU impregnated discs (8.83 % [range 1-24 %]) at all
time points ($P<0.001$).

Figure 7.1 Level of cell metabolism as measured by XTT-assay in *C. albicans*
(ATCC 90028) biofilms grown on chlorhexidine impregnated acrylic discs,
fluconazole impregnated discs or control discs after 2-28 days incubation. The
colour intensity of formazan salt produced by sessile cells constituting the
biofilm is directly correlated to cellular metabolic activity of the biofilm. Bars
represent the mean of five parallel discs.
Figure 7.2  Quantity of biomass as measured by crystal violet staining in *C. albicans* (ATCC 90028) biofilms grown on chlorhexidine impregnated acrylic discs or fluconazole impregnated discs or control discs after 2-28 days incubation. The results presented as OD where the biofilm biomass is proportional to the absorbance values. Bars represent the mean of five parallel discs.
7.5 Discussion

The results of the present study show that chlorhexidine impregnated PEM/THFM acrylic has remarkable anti-candidal biofilm activity, which is demonstrated by its ability to prevent biofilm biomass growth and biofilm metabolism up to 28 days. In contrast, fluconazole impregnated discs inhibited biofilm formation on the discs poorly, with a maximum of 40 % reduction in biofilm metabolic activity at two days: after this, activity was only marginally better than that of the drug-free control. These results are in line with those of a previous study where chlorhexidine coating of acrylic discs was used (Meiller et al., 2001). In the present study, acrylic discs impregnated with antimicrobials were used as a drug carrier to evaluate their ability to prevent candidal colonisation and biofilm formation. Instead of preformed biofilms planktonic candidal cells were allowed to adhere on the drug-impregnated discs. This model has been shown to enable leaching of fungicidal amounts of impregnated drugs and the leachates to be effective against planktonic cells (Salim et al., 2012a).

Biofilm maturation is an active process requiring metabolic activity. Chlorhexidine impregnation almost completely inhibited the metabolic activity of the adhered candidal cells for at least 28 days (study end point). Adhesion and some cell division did, however, take place as reflected by the slow increase in biomass over time. It has previously been reported that 8 × MIC for chlorhexidine is required to inhibit preformed C. albicans biofilms (Lamfon et al., 2004). Our previous observations reported that over 200 × MIC of the planktonic C. albicans cells become leached during the first week of incubation using this model (Salim et al., 2012a). The high concentrations of chlorhexidine leaching from the drug carrier may disrupt biofilm
formation at all steps from adherence through development to maturation. It has been shown that chlorhexidine reduces the adherence of *C. albicans* to surfaces (Jones *et al.*, 1997; Pizzo *et al.*, 2001), suppresses candidal hyphal formation (Kimura and Pearsall, 1980; Hazen *et al.*, 1991; Ellepola and Samaranayake, 2000b) and significantly reduces cell surface hydrophobicity (Anil *et al.*, 2001). The exceptional efficiency of CHX impregnated discs reported in this study is in agreement with previous studies where CHX showed up to 100% reduction in the activity of pre-formed biofilm (Redding *et al.*, 2009; Ramage *et al.*, 2011). In another study 30 s exposure to chlorhexidine resulted in 99.6% reduction in *Candida* biofilm formation (Meiller *et al.*, 2001).

Fluconazole impregnation inhibited biofilm formation only marginally. This is surprising as high concentrations (>1400 mg/L) of fluconazole become leached from this drug carrier (Salim *et al.*, 2012a). This concentration is 1300 times that of the MIC of the planktonic form of the *C. albicans* used in this study, which is interesting given that planktonic cells were used as the initial inoculum. However, these results are in line with previous studies on the efficacy of fluconazole on preformed biofilms where the MIC of the sessile form was >128 mg/L (Ramage *et al.*, 2011), and this sessile MIC has been reported to be up to 4000 times that of planktonic *C. albicans* cells (Ramage *et al.*, 2004). This innate resistance has been explained by different mechanisms such as upregulation of efflux pumps and increased cell density (Ramage *et al.*, 2005). It is of interest if these mechanisms are active in cells ‘planning’ to form a biofilm. It is also possible that there is enough delay before fungicidal concentrations are reached in the leachates. However, in our previous study the highest concentrations leached initially and gradually decreased overtime.
Nonetheless the anti-biofilm activity profile for the CHX and FLU impregnated discs correlated well with the leached concentrations detected for CHX and FLU which decline overtime during 28-day incubation in our previous study (Salim et al., 2012a).
7.6 Conclusion

The findings of the current study demonstrate that chlorhexidine has excellent anti-biofilm activity, which is superior to that of fluconazole for an extended time period. This work, together with the previously shown excellent activity of chlorhexidine against candidal biofilms, reinforces the use of chlorhexidine for the treatment of oral candidosis. The chlorhexidine impregnated PEM/THFM polymeric drug carrier system could potentially be used as a denture liner for the treatment of denture-induced stomatitis, a difficult-to-treat candidal-bacterial biofilm infection with high relapse rate. Sub-therapeutic exposure of Candida biofilms to azoles, a critical attribute in emergence of resistance, could be avoided. These promising in vitro results warrant confirmation in vivo. In addition, these results highlight the poor activity of fluconazole against biofilms and question its efficacy for prophylaxis in patients at risk of systemic candidosis and with medical devices at risk of Candida biofilm infection.
CHAPTER 8

Impregnation with antimicrobials challenge bonding properties and water sorption behaviour of an acrylic liner

Nesreen Salim, Julian D. Satterthwaite, Riina Rautemaa, Nick Silikas

8.1 Abstract

Objectives: To investigate the effect of impregnation of poly (ethylenmethacrylate) and tetrahydrofurfuryl methacrylate (PEM/THFM) polymeric delivery system with chlorhexidine or fluconazole on shear bond strength (SBS) and water sorption.

Methods: For SBS testing, 16 PEM/THFM discs impregnated with chlorhexidine (CHX), pure fluconazole (FLUp) or fluconazole from capsules (FLUc) and 16 drug-free control discs were prepared and bonded to heat-cured acrylic blocks. All discs were allowed to set for 24 h at room temperature. After setting, half the discs (n=8) were tested immediately (Group 1). The other half was further incubated in water for 28 days at 37 °C before testing (Group 2). To evaluate water uptake, five PEM/THFM discs impregnated with CHX, FLUp or FLUc and five drug-free control discs were prepared and incubated in water. Mass changes were measured up to 6 months.

Results: The mean SBS for control, FLUp, CHX and FLUc discs were 4.01, 3.85, 3.29 and 2.26 MPa, respectively for Group 1. Group 2 showed significantly lower SBS (P≤0.05). All failures were predominantly adhesive. The percentage mass change due to water sorption ranged significantly from 12 % for control to 27 % for FLUc (P≤0.05). A strong negative correlation between the extent of water absorption and the SBS was detected (r=0.94, P=0.05).

Significance: Impregnation with antimicrobials presents a challenge to the physical and mechanical properties of a polymer. However, despite increased water uptake SBS remained acceptable for a temporary lining material and comparable to drug-free long-term lining materials. Moreover, the enhanced water uptake could contribute to improved leaching.
8.2 Introduction

A wide range of drug delivery systems has been suggested to be used for the treatment of many oral conditions by incorporation of antimicrobial agents (Patel et al., 2001; Amin et al., 2009) with denture acrylic resin or with soft liners. Using polymerised acrylic as a carrier for drugs orally is a method to extend the duration of effective therapy (Douglas, 1977; Darwish et al., 2011). The use of drug carriers is convenient for patients as they do not require compliance for frequent application regimes. In addition, direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug-drug interactions (Douglas, 1977). Incorporation of different antifungal agents in polymeric systems used in dentures has shown remarkable efficiency (Patel et al., 2001; Amin et al., 2009; Salim et al., 2012a).

Various polymeric systems have been suggested for the controlled release of bioactive agents in the oral cavity (Addy, 1981; Amin et al., 2009). Many of these systems are based on poly (methyl methacrylate) acrylic systems (PMMA) (Brook and Van Noort, 1985). Self-curing systems based on poly (ethylmethacrylate)/tetrahydrofurfurylmethacrylate (PEM/THFM) have superior characteristics compared with methyl methacrylate and n-butyl methacrylate based systems, where it has low exothermic reaction compared to the former and superior biological properties compared to the latter (Patel and Braden, 1991a; Patel et al., 1994). This system was reported to be a ductile material, which is a good attribute for prostheses (Patel and Braden, 1991c). Moreover, the same system was documented to enjoy excellent biological properties such as supporting growth and
viability of chondrocytes and being non-irritant in dental use (Pearson et al., 1986; Wyre and Downes, 2000).

Mechanical properties such as flexural strength, hardness, and strong bonding to the underlying substrate are vital characteristics for intra-oral liners and prostheses (Polyzois and Frangou, 2002; Mese and Guzel, 2008). A strong bond between the medicated lining and underlying acrylic denture is also critical for safe durable and effective delivery of the drug for the required treatment period (Maeda et al., 2012). However, the incorporation of drugs into the acrylic may compromise its mechanical and physical properties (Addy and Handley, 1981). A marked reduction in compressive and tensile strength has been demonstrated in bone cements containing more than 8 % of antibiotics (Lautenschlager et al., 1976). Equally, soft linings impregnated with nystatin showed a noticeable increase in water uptake (Douglas and Clarke, 1975). Many studies have investigated the bond strength of liners bonded to denture base resin using different tests such as tensile, shear or peel tests (Ribeiro Pinto et al., 2002; Mese and Guzel, 2008; Hatamleh and Watts, 2010; Maeda et al., 2012). However, less is known about the mechanical and physical properties of the PEM/THFM polymeric system, and the impact of incorporation of antimicrobial agents on its different properties has not been evaluated.

In view of the limited research in this area, this study was set out to assess whether incorporation of antimicrobial agents compromises the clinical performance and the properties of the PEM/THFM acrylic material. The aims of this study were: 1) to investigate the shear bond strength of PEM/THFM containing chlorhexidine and fluconazole to heat cured acrylic substrate in standard conditions as well as after storage in distilled water, and 2) to investigate the water uptake of PEM/THFM
containing chlorhexidine and fluconazole. The null hypotheses were that 1) the bond strength would not be significantly affected by impregnating the PEM/THFM acrylic system with antimicrobial agents, and 2) the water uptake behaviour of the drug-free PEM/THFM discs is not significantly different compared to the impregnated discs.
8.3 Materials and methods

Study Design

The impact of incorporation of chlorhexidine (CHX), pure fluconazole (FLUp) or fluconazole powder from capsules (FLUc) on shear bond strength (SBS) and water sorption of poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) was tested in vitro. For shear bond strength, 16 parallel PEM/THFM discs were prepared by replacing a proportion of the PEM by CHX, FLUp or FLUc (described in detail below). Sixteen control discs were prepared following manufacturer’s instructions. All discs at dough stage were bonded to heat-cured acrylic blocks and were allowed to cure for 24 h (the time for complete setting). Then the discs were allocated into two different groups (n=8): Group 1 discs were tested immediately and in Group 2 discs were further stored in water for up to 28 days at 37 °C. All specimens were tested using shear bond strength test. For water sorption analyses, a total of 20 discs were prepared. Of these, 15 PEM/THFM discs were prepared by replacing a proportion of the PEM by CHX, FLUp or FLUc. Five control discs were prepared following manufacturer’s instructions. All discs were incubated in water at 37 °C and mass changes were measured for up to 6 months.

Shear Bond Strength

For shear bond strength (SBS) testing, a heat cure acrylic resin (Skillbond Ltd, Bucks, UK) was prepared following manufacturer’s instructions (Table 8.1). Powder and liquid were mixed in a 3:1 ratio until plastic dough was obtained. After mixing the material was packed inside a hollow cylindrical brass mould (external diameter = 18 mm, internal diameter = 16 mm, depth = 25 mm). A 6 h long cure cycle was applied using a 95 °C hot water bath (the first 4 h at 60 °C). The surfaces were
prepared for bonding 24 h after fabrication by rubbing with 60 grit silicone carbide waterproof abrasive paper. The acrylic surfaces were wiped with acetone and left to dry. Subsequently, a thin and homogenous layer of the primer (Principality Medical Ltd, Newport, UK) was applied with a brush onto the surface, and the surfaces were left to dry for 30 min at room temperature according to the manufacturer's instructions.

Table 8.1 Groups of the shear bond study: control, chlorhexidine (CHX), fluconazole pure (FLUp) and fluconazole from capsules (FLUc)

<table>
<thead>
<tr>
<th>groups (n=16)</th>
<th>powder/liquid ratio</th>
<th>Acrylic substrate</th>
<th>Acrylic lining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>3:1 heat cured PMMA</td>
<td>1:0.6 PEM/THFM</td>
<td></td>
</tr>
<tr>
<td>CHX group</td>
<td>3:1 heat cured PMMA</td>
<td>1:0.6 PEM/THFM with 10 % by weight of CHX substituted in powder</td>
<td></td>
</tr>
<tr>
<td>FLUp group</td>
<td>3:1 heat cured PMMA</td>
<td>1:0.6 PEM/THFM with 10 % by weight of FLUp substituted in powder</td>
<td></td>
</tr>
<tr>
<td>FLUc group</td>
<td>3:1 heat cured PMMA</td>
<td>1:0.6 PEM/THFM with 25 % by weight of FLUc substituted in powder</td>
<td></td>
</tr>
</tbody>
</table>

To prepare the drug-free control discs (n=16), a cold cure-polymerised acrylic resin (PEM/THFM) was prepared using ratio of 1 g of PEM powder (Lucite International, Durham, UK) to 0.6 ml of THFM liquid (Sigma-Aldrich, Dorset, UK). Materials used in this study are presented in Table 8.1. Three medicated groups were prepared (n=16). The drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed: 10 % (w/w) of the PEM was replaced with CHX (Sigma-Aldrich) 10 %
(w/w) by FLUp (Sigma-Aldrich) or 25 % (w/w) by FLUc (Pfizer, Kent, UK) (resulting in 100 mg of active drug per disc, for all). Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25 % of it is equivalent to 10 % of pure fluconazole, as confirmed by bioassay and minimum inhibitory concentration testing (MIC). The PEM/THFM mixtures of all groups were packed against the primed surface in the dough stage using teflon discs (external diameter= 18 mm, internal diameter = 8 mm, thickness = 3 mm) to produce a constant area of bonding for all samples (Figure 8.1).

![Image](image_url)

**Figure 8.1** A photograph showing the heat cure acrylic substrate inside the cylinder and the cured PEM/THFM specimen attached to the acrylic substrate and showing the adhesive bond failure after subjected to shear bond test

After 24 h curing of all samples, samples were allocated into two different main groups (n=8); Group 1 discs were tested immediately and in Group 2 discs were further stored in water for 28 days at 37 °C. For water storage, samples were separately immersed in 100 ml distilled water and left in a thermostatically controlled cabinet at 37 °C for 28 days. All samples were debonded using a shear test
The shear jig complies with ISO/TR 11405:1994 standard (International Organization for Standardization, 1994). Cross head speed was set at 1mm/min. The force-to-failure was recorded (N) and the type of bond failure was determined and classified as adhesive, cohesive or combined failure (Figure 8.1). Examination of the specimens for adhesive or cohesive failures was carried out and judged by two examiners. The shear bond strength (SBS) was calculated according to the following equation:

\[
\text{Eq.}(8.1)
\]

Where \( F \) stands for maximum force to failure expressed in (N), \( A \) is the area of attachment expressed in (mm\(^2\)); SBS represents the bond strength (MPa). Data are presented as means of SBS and standard deviations.

**Water Sorption**

For water sorption investigation, the control discs were prepared by mixing 5 g of PEM and 3 ml of THFM. For the impregnated discs, the drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed as described above for SBS. All mixtures were packed into disc shaped steel moulds (40 mm diameter and 0.5 mm height) and allowed to cure for 15 min.

All discs (n=5) were stored in desiccators with anhydrous silica gel at 37 °C and weighed to ± 0.01 mg regularly using a calibrated electronic analytical balance with a precision of 0.01 mg (Ohaus Analytical Plus, Ohaus Corporation, USA), until the mass change of each disc was constant and not more than 0.1 mg in any 24 h period this
was to ensure complete dehydration. This constant mass is the baseline mass \( (m_1) \) for the discs. The discs were then soaked individually in 100 ml of sterile distilled water at 37 °C in tightly sealed sterile plastic containers. Readings were taken daily for the first week and then weekly up to two months and then every month up to 6 months. For each measurement samples were carefully removed from the water with tweezers, dried on filter paper until free from visible moisture, air-dried for 15 s and weighed and returned to the water bath. The recorded mass at any given time was denoted as \( (m_2) \). The percentage apparent mass change \( (M_g) \) was calculated by:

\[
\text{Eq. (8.2)}
\]

The data were presented as mean percentage of mass change.

**Statistical Analysis**

The bond strength results and water sorption for all groups were analysed by one-way ANOVA and Dunnett T3 *post hoc* test at 0.05 significance level. Modes of failure were decided visually by two examiners. Linear regression was used to test the correlation between water sorption and bond strength and was used to test the correlation between water uptake measured in this study and amount drug leached reported previously (Salim *et al.*, 2012a).
8.4 Results

Mean shear bond strengths for all groups are shown in Table 8.2. For Group 1 (tested immediately), the control discs of cured PEM/THFM resin revealed the highest SBS (4.01 MPa) which was significantly higher compared to SBS of FLUc impregnated discs ($P \leq 0.001$) and CHX impregnated discs ($P = 0.046$), but not for FLUp ($P = 1.00$). The difference was not statistically significant between FLUp discs (3.85 MPa) and CHX discs (3.29 MPa) ($P = 0.47$). After water incubation for 28 days, the control discs of cured PEM/THFM showed the highest bond strength (2.89 MPa) with a significant difference between all stored specimens ($P \leq 0.001$). The SBS were significantly lower in all water-stored specimens (Group 2) compared to their corresponding Group1 specimens ($P \leq 0.05$, for all) (Figure 8.2). The mode of failure was adhesive at the lining/base interface in all specimens (Figure 8.1).

Table 8.2 Mean (SD) of shear bond strengths (MPa) for all discs: Group 1 (tested immediately) and Group 2 water-stored discs (water immersion for 28 days). These groups consisting of control discs without impregnation, discs impregnated with pure fluconazole (FLUp), chlorhexidine (CHX), and fluconazole from capsules (FLUc) (different upper case alphabets in rows indicate significant difference at $P \leq 0.05$)

<table>
<thead>
<tr>
<th>Tested groups</th>
<th>Sub-groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Group 1</td>
<td>4.01(0.47)$^A$</td>
</tr>
<tr>
<td>Group 2</td>
<td>2.89(0.33)$^A$</td>
</tr>
</tbody>
</table>
Figure 8.2 A bar chart showing the mean shear bond strength (standard deviation) under two different conditions: 1) the specimens stored dry at room temperature (Group 1) to be tested after 24 h, and it comprises 4 groups: control discs, discs impregnated with chlorhexidine (CHX), pure fluconazole (FLUp) or fluconazole from capsules (FLUc). 2) The specimens were further immersed in water at 37 °C for 28 days after 24 h setting (Group 2) and it consists of the same sub-groups of discs. Horizontal capped lines indicate significant difference between the dry stored discs and their corresponding water stored discs ($P \leq 0.05$)

Figure 8.3 shows the mean percentage of mass change against square root of time ($t^{\frac{1}{2}}$) of PEM/THFM with the presence and absence of the antifungal agents after immersion in water for up to 6 months. A significant increase in mass after water storage was detected compared to the initial mass for all discs ($P \leq 0.05$). The control absorbed 12 % water compared to 19 %, 23 % and 27 % for FLUp, CHX and FLUc impregnated discs respectively after 6 months. The mass change was significantly different for the control discs when compared to FLUp, CHX and FLUc ($P=0.004$, $P=0.001$, $P \leq 0.001$ respectively) after 6 months. After 28 days of water absorption the...
mass change between all groups was significantly different ($P \leq 0.001$). PEM/THFM did not equilibrate after 6 months.

![Graph showing the percentage mass change of control discs, discs impregnated with chlorhexidine (CHX), fluconazole from capsules (FLUc) and pure fluconazole (FLUp) immersed in distilled water for 6 months (510.5 min$^{1/2}$).](image)

**Figure 8.3** Percentage mass change of control discs, discs impregnated with chlorhexidine (CHX), fluconazole from capsules (FLUc) and pure fluconazole (FLUp) immersed in distilled water for 6 months (510.5 min$^{1/2}$)

The correlation between mass change and square root of time in the early stages of water uptake was linear reflecting a diffusion-controlled uptake. A strong negative correlation between the water absorption and SBS was demonstrated ($r=0.94$, $P=0.05$). In addition, a significant positive correlation between water uptake measured in this study and amount drug leached reported previously was detected for all drugs: ($r=0.99$, $P \leq 0.001$) for CHX, ($r=0.876$, $P=0.022$) for FLUp and ($r=0.817$, $P=0.047$) for FLUc (Salim *et al.*, 2012a).
8.5 Discussion

It has been shown previously by the authors of the current study that microbiologically efficient levels are leached from PEM/THFM polymeric system impregnated with fluconazole or chlorhexidine (Salim et al., 2012a). The results of the present study show that the impregnation of PEM/THFM with chlorhexidine or two formulations of fluconazole has a significant impact on their water uptake and bond strength to heat cured acrylic substrate. Consequently, both null hypotheses were rejected. However, the detected bond strength is to be considered sufficient and durable to service (>0.44 MPa) (Craig and Gibbons, 1961; Kawano et al., 1992; Ribeiro Pinto et al., 2002). In addition, even the lowest values seen with FLUc impregnated discs exceeded that of long-term lining materials (>0.7 MPa) (Hatamleh and Watts, 2008). There are no previous reports exploring the SBS and the water uptake of the PEM/THFM system when impregnated with antimicrobials. Importantly, the impact of the three incorporated agents was found to be different. Fluconazole from capsules showed a more adverse impact on SBS and water uptake compared to both fluconazole pure and chlorhexidine.

The polymeric system used in this study showed a remarkable water absorbing capability compared to other similar polymeric systems (Doğan et al., 1995). This could be beneficial for a drug delivery system and may result in enhanced drug release. However, the possibility of leaching residual monomer increases as the water absorption increases (Jagger, 1978). Nevertheless, this polymeric system is suggested to be used for a temporary use with well documented biologically satisfactory characteristics (Pearson et al., 1986; Sawtell et al., 1995). Interestingly, a significant positive correlation between the water uptake measured in this study and the
amount drug leached reported previously was detected for all drugs. However, water uptake does not necessarily reflect the leached drug levels if the compound contains impurities and excipients like FLUc.

The increased water uptake in the impregnated discs could be due to the formation of droplets around the impregnated drug particles which will enlarge as the water is diffusing in due to the osmotic gradient between the internal droplet and the external solution (Riggs et al., 2000). Another factor that may play a role in the water uptake is the solubility of the impregnated drug. While pure fluconazole has a limited solubility (5 mg/ml at 37°C), chlorhexidine is characterised by higher water solubility (19 mg/ml at 37 °C) compared to fluconazole. Moreover, the molecular weight for CHX is twice as much as the molecular weight of FLU (Figure 8.4) which may also explain differences in their impact on water absorption; as the CHX particles leach out of the polymer they will leave larger voids behind absorbing more water compared to the voids created as a result of fluconazole leaching.

![Figure 8.4 Molecular structure of fluconazole (a) and chlorhexidine (b)](image-url)
Bond strengths of PEM/THFM to heat cured PMMA were influenced by impregnating the former with antimicrobial agents under standard and conditioned circumstances. This could be related to the presence of the antimicrobials which may disrupt the polymer structure and introduce more spaces and less homogeneity in the resin matrix and thus potentially weaken the bond strength between the doped lining and the denture. Moreover, the agents may interfere with the polymerisation, precluding further propagation of the chain (Riggs et al., 2000). All bond strength failures were adhesive failures. This indicates that the bond strength between the lining and the acrylic is weaker than the lining strength which is an advantage for a temporary lining in practice. The further reduction in the bond strength after immersion in water can be explained by the distinctive water absorption capability of the PEM/THFM polymer. This finding is reflected by the strong negative correlation between the bond strength and the water absorption capability (Riggs et al., 1999; Riggs et al., 2000). This finding is in line with other studies that tested different resilient liners (Mese and Guzel, 2008).

Significant differences were detected between the influence of pure fluconazole and fluconazole powder from capsules. This is likely to be due to the presence of excipients in the powder from capsules and the need to replace a higher proportion of the PEM powder in order to incorporate equivalent amount of active drug into the discs. Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25 % of it is equivalent to 10 % of pure fluconazole, as confirmed by bioassay and MIC testing (Salim et al., 2012a). This may disrupt the polymer structure and produce more porous structure which reflects weaker bond strength and higher sorption ability. The formulations of fluconazole used
in previous studies were not clearly defined therefore both formulations were included in this study. Fluconazole capsules are readily available to dental practitioners and are the commonly prescribed form which worth to be investigated in this study.

The storage period for SBS was decided based on the average time suggested in the literature for the drug delivery device usage (Patel et al., 2001; Amin et al., 2009). The samples were incubated in distilled water at 37 °C to simulate the clinical situation. The water absorption from biological fluids such as saliva is much lower than that in distilled water as a result of the increased osmolarity which limits the water uptake. Consequently the bond strength can be expected to be higher in the oral cavity than that tested in distilled water in vitro (Riggs et al., 1999; Hutcheon et al., 2001). The shear bond test used in the present study does not represent the sum of acting forces in the oral cavity. However, it relates well to the loads that affect the lining materials during function (Hatamleh and Watts, 2010) and it does offer useful information on how a modification of the lining material may affect its mechanical properties (Hatamleh and Watts, 2010). Importantly, all antifungal drugs and formulations that were tested in the current study showed excellent antifungal activity after being leached from PEM/THFM in our previous study (Salim et al., 2012a). These findings reinforce the importance of testing the bond strength and water absorption characteristics to investigate the serviceability and durability of the suggested delivery system in clinical use. Further studies are warranted to investigate the usability and efficiency of this drug delivery system in vivo.
8.6 Conclusions

Within the limitations of this *in vitro* work, the following conclusions may be drawn:

1- Incorporating antifungal agents into PEM/THFM resin by polymer substitution significantly affects its bond strength to acrylic substrate and the water uptake characteristics.

2- Immersing samples in water has a significant influence on the bond strength as a result of distinctive water absorption ability of the PEM/THFM.

3- Despite the reduction in the bond strengths after water storage, all bonds are still serviceable and clinically durable to be used as a long-term lining for effective therapy.

4- The mode of failure was adhesive in all specimens.
CHAPTER 9

Impregnation with antimicrobials has an impact on degree of conversion and colour stability of acrylic liner

Nesreen Salim, Julian D.Satterthwaite, Riina Rautemaa, Nick Silikas

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9.1 Abstract

This study investigated the impact of impregnation of a poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) polymer with chlorhexidine or fluconazole on the degree of conversion (DC) and colour stability (ΔE*). The DC of uncured (0 h) and cured (24 h) samples was analysed by fourier transform infrared spectroscopy (FTIR) and colour stability was analysed colorimetrically. The DC percentage of the control samples was significantly greater than those containing chlorhexidine and fluconazole (P≤0.05). The control discs exhibited only slight colour change compared to the impregnated discs which showed marked colour change (P≤0.05). A strong negative correlation between the extent of colour change and the degree of conversion was detected (r=0.97). The DC and colour stability were influenced by the addition of chlorhexidine or fluconazole. However, the final values were comparable to other commonly used acrylic liners and within acceptable ranges. PEM/THFM can be considered as a biocompatible drug delivery system.

**Key Words:** Chlorhexidine, Fluconazole, Degree of Conversion, Colour change, PEM/THFM acrylic liner.
9.2 Introduction

Polymeric systems enabling controlled drug-release have been suggested for a range of dental therapeutic applications (Wyre and Downes, 2000; Patel et al., 2001; Ryalat et al., 2011). A prolonged therapeutic effect can be achieved using such delivery systems whereby high conventional systemic doses can be replaced by slow local release of lower doses (Douglas, 1977). Several systems have been suggested to be used as intra-oral delivery devices (Riggs et al., 2000; Ryalat et al., 2011). These systems are predominantly based on poly (methylmethacrylate) (PMMA) (Amin et al., 2009; Ryalat et al., 2011). A number of bioactive delivery systems based on a cold-cure poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) polymer system have been introduced with promising results (Riggs et al., 2000; Wyre and Downes, 2000; Patel et al., 2001; Salim et al., 2012a). This polymer can be used as a denture lining material or as a base for dental appliances. There are some studies on its physical and chemical properties as well as biocompatibility (Pearson et al., 1986; Patel et al., 1987; Patel and Braden, 1991c). It has been suggested that impregnating PEM/THFM with an antimicrobial such as chlorhexidine (CHX) may influence the polymerisation and the degree of conversion (DC) of the polymer which may have an adverse impact on its mechanical properties (Riggs et al., 2000).

Ideal polymerisation of acrylic resins should result in conversion of all monomer into polymer. However, in practice, some residual monomer can always be found in the polymer (Bural et al., 2011). Slow leaching of excess monomer into saliva can cause local irritation and development of hypersensitivity (Baker et al., 1988). The level of residual monomer is related to the DC and polymerisation which can be achieved
using chemical activators in autopolymerising acrylic resin (Vallittu et al., 1995; Vallittu, 1996). Consequently, DC is a critical parameter that influences the biocompatibility and the mechanical and physical properties of dental polymers (Baker et al., 1988; Boeckler et al., 2008) and assessing the DC is a valuable tool for evaluating the mechanical properties of a polymer and its clinical performance.

Various techniques have been employed to measure the level of polymerisation and DC including high performance liquid chromatography (HPLC), spectroscopy and high precision measurement of the extent of polymerisation shrinkage (Vallittu et al., 1995; Duray et al., 1997). Spectroscopic analysis is extensively used in dentistry given that it is a specific, economic, adequately sensitive technique and provides a reliable quantitative measure to assess conversion (Duray et al., 1997). Fourier Transform Infrared Spectroscopy (FTIR) is a powerful analytical technique that has been utilised as a quantitative measure for identification and monitoring setting reactions and polymerisation of a broad range of dental materials (Guerra et al., 1996; Silikas et al., 2000). The basic functioning of FTIR relies on infrared radiation emitted from a heated material which interacts with a substance under investigation: this interaction is measured and analysed by a highly sensitive spectrometer (Celina et al., 1997).

Colour stability is another significant clinical parameter for dental materials (Anil et al., 1999). The colour stability of chemically activated acrylic resins is less satisfactory compared to conventional heat cured acrylic resin (May et al., 1992). This has been suggested to be related to the higher level of residual monomer and the breakdown of benzoyl peroxide and tertiary amine constituents leading to discolouration (Purnaveja et al., 1982). Colour change reflects the water absorption
of the liner thus it is an important parameter to evaluate the durability of the liner (Ergun and Nagas, 2007). Colour stability also appears to reflect the residual monomer content: the higher the residual monomer the more the colour change (Austin and Basker, 1982; May et al., 1992).

A number of studies have investigated the degree of conversion and colour stability of PMMA but little is known of these two properties for PEM/THFM. Even less is known of the effect of incorporation of bioactive agents on these properties. In view of the scarce research in this area, this study was carried out to evaluate whether incorporation of antimicrobial agents has an impact on these properties of the PEM/THFM acrylic material. Therefore, the aim of this study was to investigate the effect of impregnation of chlorhexidine or fluconazole on the degree of conversion and colour stability of a PEM/THFM polymeric system. The null hypotheses were: 1) there is no significant difference in the degree of conversion between the drug-free PEM/THFM discs and the impregnated discs and 2) there is no significant difference in the colour stability between the tested groups.
9.3 Materials and methods

Study Design

The impact of incorporation of chlorhexidine (CHX), pure fluconazole (FLUp) or fluconazole powder from capsules (FLUc) on degree of conversion and colour stability of poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) was tested in vitro. For degree of conversion (DC), a total of 40 PEM/THFM samples were prepared. Ten PEM/THFM mixtures for each test group were prepared by replacing a proportion of the PEM by CHX, FLUp or FLUc. Ten control mixtures were prepared following manufacturer’s instructions. The DC of uncured (0 h) and cured (24 h) samples was analysed by Fourier Transform Infrared Spectroscopy (FTIR) and the DC was calculated. For colour stability, a total of 20 discs were prepared. Of these, fifteen PEM/THFM discs were prepared by replacing a proportion of the PEM by CHX, FLUp or FLUc (five discs in each group). Five control discs (CTR) were prepared following manufacturer’s instructions. The discs were allowed to cure for 24 h and then incubated in water at 37 °C for 28 days. The colour was recorded at baseline and after 28-day incubation period and colour change ΔE*ab was calculated. Colour stability was analysed colorimetrically using the National Bureau of Standards 6-scale rating.

Sample Preparation

To prepare the control samples, 1 g of PEM (Lucite International, Durham, UK) and 0.6 ml of THFM (Sigma-Aldrich, Dorset, UK) were mixed. The powder contained <1 % benzoyl peroxide (initiator) and N,N-dimethyl-p-toluidine (Sigma-Aldrich) was used as an activator. For the impregnated samples, the drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed: 10 % (w/w) of the PEM was
replaced with CHX (Sigma-Aldrich) or 10 % (w/w) by FLUp (Sigma-Aldrich) or 25 % (w/w) by FLUc (Pfizer, Kent, UK) (resulting in 100 mg of active drug per disc, for all). Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25 % of it is equivalent to 10 % of pure fluconazole, as confirmed by bioassay and minimum inhibitory concentration testing (MIC). For DC, mixtures were stirred until plastic dough was obtained and then the freshly mixed uncured acrylic was distributed on the surface of the detector crystal of the FTIR and covered to prevent light interference. For colour stability, mixtures were packed into disc shaped steel moulds (10 mm diameter and 1 mm height) and allowed to cure for 15 min as per manufacturer’s instructions.

**FTIR Spectroscopy**

The DC for all samples was investigated using the FTIR spectrometer Avatar™ 360 (Thermo Fisher Scientific, Surrey, UK). Micro-attenuated total reflectance was used (micro-ATR), which is a well established method (Niepraschk et al., 2007). The FTIR was used under the following conditions: 4000-500 cm⁻¹ range, 4 cm⁻¹ resolution, 32 scans coaddition. Initially, a background FTIR spectrum without a sample was collected. Then a spectrum was recorded for the uncured samples after which the samples were left to set. Another FTIR spectrum was collected for the same samples after 24 h curing (the time for complete setting).

PEM/THFM is a chemical-cured acrylic and its polymerisation progresses via free-radical addition across aliphatic double bonds. As reaction proceeds, DC can be calculated by measuring the decrease of aliphatic bonds (C=C) at 1635 cm⁻¹ versus carbonyl bonds (C=O) (a constant internal standard) at 1720 cm⁻¹ before and after
polymerisation. This method of calculation is called internal standard method to evaluate the residual monomer concentration according to following formula:

\[
X = \frac{\text{absorption of aliphatic}}{\text{absorption of carbonyl}}_{\text{cured}}
\]

\[
y = \frac{\text{absorption of aliphatic}}{\text{absorption of carbonyl}}_{\text{uncured}}
\]

**Colour Stability**

Discs (n=5 per group) were soaked individually after 24 h of preparation in 10 ml of sterile distilled water at 37 °C in tightly sealed glass vials. The containers were gently shaken three times daily throughout the experiment to simulate the clinical situation. The colour of all specimens was measured at baseline before storage and subsequently after 28 days water storage. All measurements were carried out using a colorimeter (Minolta Chroma Meter CR-221, Osaka, Japan), according to the CIE L*a*b* system (Commission Internationale d’Eclairage). This system describes three colour values of L*, a* and b*, to facilitate classifying colour differences numerically. The device was set to measure the 3-dimensions of the colour (L*a*b*). The L* parameter reflects the degree of lightness and darkness which ranges from black (0) to white (100), whereas a* and b* are chromacity which indicate red or green chroma (+a* = red, -a* = green) and yellow or blue chroma (+b* = yellow, -b* = blue) correspondingly. Each specimen was positioned against a white-tile ceramic background under the same lighting conditions of average daylight illumination (CIE illuminant D65); the measuring area was 3 mm and 45° illumination angle and 0° viewing angle. Specimens were notched and placed into a positioning disc to
measure the colour at the same location for all specimens before and after water storage. Measurements were repeated three times and means for L*, a*, and b* values were calculated. The colour changes, ∆E*ab, were calculated from the three colour values L*, a* and b* according to the following formula:

\[
\Delta E^*_{ab} = \sqrt{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2}
\]

Where:

\((L^*_1, a^*_1, b^*_1)\) the values at baseline
\((L^*_2, a^*_2, b^*_2)\) after 28 days time interval

Colour changes (\(\Delta E^{*ab}\)) were further quantified using the National Bureau of Standards (NBS) with NBS units of colour difference which reflect human eye evaluation of colour change (Yaman et al., 1989; Shotwell et al., 1992; Ergun and Nagas, 2007). The values of NBS units were calculated using the following formula:

\[
\text{Eq...(9.3)}
\]

Data are presented as means of colour change with standard deviation and means of L*, a*, b* values.

**Statistical Analysis**

A one-way analysis of variance (ANOVA) was applied to investigate the significant differences in degree of conversion and colour change between groups (\(P \leq 0.05\)). To check the assumption of equal variances, all data were subjected to Levene's test of homogeneity of variance (\(\alpha = 0.05\)). Equal variances assumption was accepted for DC
data and Bonferroni multiple comparison test was used for post hoc analyses. Equal variances assumption was rejected for colour stability data \((P \leq 0.05)\) and consequently Dunnett’s T3 multiple comparison test was applied to compare the colour change \((\Delta E^{*ab})\) as well as the change in colour values \((\Delta L^*, \Delta a^*, \Delta b^*)\) between the tested groups. A Paired T-test was used to compare the change in colour values \((\Delta L^*, \Delta a^*, \Delta b^*)\) within each group. Linear regression was used to test the correlation between the degree of conversion and colour stability. Linear regression was used also to test the correlations between DC and \(\Delta E^{*ab}\) measured in this study with water uptake reported previously (Salim et al., 2012b).
9.4 Results

Mean percentages of degree of conversion for all tested discs after 24 h of cure are illustrated in Table 9.1. The PEM/THFM drug-free control discs (CTR) exhibited a significantly higher DC % than all impregnated discs ($P \leq 0.05$) except FLUp discs where the difference was not significant ($P = 0.062$). The FLUc impregnated discs demonstrated the lowest DC %, which was markedly lower than DC % detected for all other tested discs ($P \leq 0.001$).

Table 9.1 Means (SD) of degree of conversion of the tested groups: Control, chlorhexidine impregnated group (CHX), fluconazole pure impregnated group (FLUp) and fluconazole from capsules impregnated groups (FLUc)

<table>
<thead>
<tr>
<th>Group</th>
<th>DC % (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>97.55a (4.61)</td>
</tr>
<tr>
<td>FLUp</td>
<td>95.82a,b (6.62)</td>
</tr>
<tr>
<td>CHX</td>
<td>95.72b (5.45)</td>
</tr>
<tr>
<td>FLUc</td>
<td>91.20c (6.20)</td>
</tr>
</tbody>
</table>

*different lower case and superscripted alphabets in same the column indicate significant difference ($P \leq 0.05$)

The carbonyl stretching band (C=O) (internal standard) was observed in the range 1720-1725 cm$^{-1}$ for the control discs without impregnation (Figure 9.1). However the carbonyl stretching band was depicted at a lower wave number for the impregnated groups compared to the control group (1719-1716 cm$^{-1}$). The stretching absorption band at 1635 cm$^{-1}$ corresponds to the carbon-carbon double bond (C=C).
Figure 9.1 A representative spectrum of the cured acrylic for all tested groups: control (a), fluconazole pure impregnated group (FLUp) (b), chlorhexidine impregnated group (CHX) (c) and fluconazole from capsules impregnated groups (FLUc) (d). The carbonyl peak corresponds to (C=O) (internal standard) where (C=C) corresponds to the aliphatic peak

The means of colour change (ΔE*ab) values and their corresponding NBS values for drug-free acrylic discs (CTR), acrylic discs impregnated with CHX, FLUp, or FLUc after water immersion are presented in Table 9.2. The highest discolouration was recorded for FLUc impregnated discs (extremely marked) (Table 9.3), which was significantly higher than all other tested groups ($P \leq 0.05$). The least discoloration was detected for free-drug control discs (slight) that was significantly lower than all other tested groups ($P \leq 0.001$). CHX and FLUp discs showed comparable ΔE*ab values, where the difference was not significant ($P = 0.410$).
Table 9.2  The mean colour changes (ΔE*ab) of the study groups and the standard deviation. The values in NBS units were calculated [ΔE*ab (in CIE) ×0.92]

<table>
<thead>
<tr>
<th>Group</th>
<th>ΔE*ab (in CIE)</th>
<th>SD</th>
<th>ΔE*ab (in NBS units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.97&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11</td>
<td>0.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FLUp</td>
<td>5.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.62</td>
<td>4.85&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHX</td>
<td>4.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45</td>
<td>4.34&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FLUc</td>
<td>9.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.20</td>
<td>9.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*different lower case and superscripted alphabets in the same column indicate significant difference (P≤0.05)

Table 9.3 National Bureau of Standard rating (NBS)

<table>
<thead>
<tr>
<th>Critical remarks of colour difference</th>
<th>NBS units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extremely slight</td>
<td>0.0-0.5</td>
</tr>
<tr>
<td>Slight</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td>Perceivable</td>
<td>1.5-3.0</td>
</tr>
<tr>
<td>Marked</td>
<td>3.0-6.0</td>
</tr>
<tr>
<td>Extremely marked</td>
<td>6.0-12.0</td>
</tr>
<tr>
<td>Change to other colour</td>
<td>&gt;12.0</td>
</tr>
</tbody>
</table>
The changes in the three colour coordinates ($L^*$, $a^*$, $b^*$) of CIE $L^*a^*b^*$ system are presented in Figure 9.2. These changes were: $L^*$ values of CTR and CHX impregnated discs increased to white after water immersion (brighter), however these values decreased (darker) after water immersion of FLUp and FLUc impregnated discs.

![Figure 9.2](image)

**Figure 9.2** The three colour values before ($L_{1}^*$, $a_{1}^*$, $b_{1}^*$) and after water immersion for 28 days ($L_{2}^*$, $a_{2}^*$, $b_{2}^*$) of drug-free acrylic discs (CTR), acrylic discs impregnated with chlorhexidine (CHX), pure fluconazole (FLUp), or fluconazole from capsules (FLUc). Horizontal lines above bars represent significant difference between paired groups $P \leq 0.05$

Significant differences were detected between all groups for $\Delta L^*$ ($P \leq 0.05$) but not between CHX and FLUp where the difference was not significant ($P = 0.079$). $a^*$ values for all tested discs increased but more intense red (higher values) measured for
FLUp and FLUc compared to CTR and CHX discs, with a significant difference between all groups (P≤0.05). b* values of the tested discs increased (more yellow) after water immersion. However, more intense yellow (higher) values were measured for FLUp and FLUc compared to CTR and CHX discs. Δb* values were significantly different between all groups (P≤0.05) but not between CHX and FLUp where the difference was not significant (P=0.360). The changes in the three values before (L*_1, a*_1, b*_1) and after storage (L*_2, a*_2, b*_2) within each group were significant (P≤0.05) except for the change of a* and b* values within the control group which was not significant (P=0.100) (Figure 9.2). A strong negative correlation between the degree of conversion and colour stability was demonstrated (r=0.97). In addition, a significant negative correlation between DC measured in this study and water absorption reported previously was detected (r=0.99, P=0.009) and a significant positive correlation between ΔE*ab measured in this study and water absorption reported previously was (r=0.97, P=0.027) (Salim et al., 2012b).
9.5 Discussion

Water incubation of PEM/THFM discs impregnated with fluconazole or chlorhexidine has been shown to result in release of highly fungicidal amounts of these drugs into the water (Salim et al., 2012a). As incorporation of drugs may impact the physical and chemical properties of the material and its serviceability, we set out to evaluate the impact of this on two important clinical properties: the degree of conversion (DC) and colour stability (Anil et al., 1999; Ergun and Nagas, 2007). Our findings demonstrate that the impregnation of antimicrobial agents can significantly influence the DC and colour stability of PEM/THFM polymeric systems and consequently both null hypotheses were rejected. However, the final values are still within the acceptable limits of ISO specification 20795-1 and better than that reported previously for other long-term lining materials (Ergun and Nagas, 2007; International Organization for Standardization, 2008).

Generally, a significant decrease in the DC of PEM/THFM was detected after impregnation with antimicrobial agents compared to the control samples. This can be attributed to the incomplete polymerisation of the polymer and therefore decreased crosslinking as a result of the presence of drug particles (Riggs et al., 2000). The crosslinking is due to ring-opening reaction of THFM monomer (Patel and Braden, 1989). The significant decrease in DC of FLUc impregnated samples compared to all other groups is related to the presence of excipients in the powder from capsules and the need to place a higher proportion into the PEM powder in order to incorporate an equivalent amount of active drug into the discs. This may disrupt the polymer structure more and further interfere with chain propagation.
Chlorhexidine influenced the efficiency of polymerisation significantly compared to the control probably due to protonation of chlorhexidine as shown by a previous study (Riggs et al., 2000). Moreover the shift in the peak corresponding to the carbonyl group in the impregnated groups compared to the control group could be attributed to hydrogen-bonding interactions with N-H groups of CHX molecules and O-H groups in FLU molecules. This finding is in agreement with a previous study which reported a shift in carbonyl group in CHX impregnated samples compared to the non impregnated samples (Gong et al., 2007). Although it is not significant, the decreased polymerisation of CHX impregnated samples compared to FLUp samples could also be related to the differences between the two molecules, since CHX has higher molecular weight. Interestingly, the polymerisation of the control group was not fully completed (DC 97.55 %) although manufacturer’s instructions were strictly followed. This could be due to the increase in viscosity as polymerisation progresses which could lead to limited mobility of the functional groups of the monomer within the polymerising structure and eventually terminating the reaction before complete conversion (Bural et al., 2011).

All investigated groups apart from the FLUc impregnated group fulfilled the ISO specification 20795-1 criteria, where the residual monomer content should not exceed 4.5 % (International Organization for Standardization, 2008). However, our study tested the whole residual monomer content within the acrylic matrix which would be higher than the leached residual monomer especially in short-term use as intended for the investigated acrylic resin (Danesh et al., 2012). Importantly, it has been proven that monomer concentration does not seem to correlate to the leaching behaviour of the monomer (Danesh et al., 2012). This could be due to the presence of
some unreacted monomers as pendant or trapped groups inside the crosslinked network.

For colour stability, PEM/THFM drug-free control discs demonstrated slight colour change compared to the colour of the impregnated groups which were affected significantly. The colour changes could be attributed to differences in water sorption, chemical composition and residual monomer (Ergun and Nagas, 2007). Interestingly, significant correlations between the DC and the colour change measured in this study and water uptake results reported previously (Salim et al., 2012b) was detected for all drugs. Accordingly, the poor colour stability of FLUc impregnated discs compared to all other groups is related to the higher water absorption and the higher amount of residual monomer as a result of incorporating a higher proportion of the FLUc powder in order to incorporate an equivalent amount of active drug into the discs. The control group showed the least colour change where the residual monomer and water absorption is lower (Salim et al., 2012b) compared to other tested groups. This finding is reflected by the strong negative correlation between the colour change and the degree of conversion. This result is in agreement with a previous study that showed a similar relationship between the amount of residual monomer and the extent of colour change for acrylic hard liners (Ergun and Nagas, 2007). Importantly, all discs showed significant differences before and after storage (within each group) in the three colour values. This can be related to the increased water absorption of PEM/THFM (Riggs et al., 2000). Although high colour change values (ΔE*ab) were measured for the impregnated PEM/THFM, it is still acceptable for short-term use as these values are within or even lower than that recorded for long-term denture liners (Ergun and Nagas, 2007).
All antimicrobial agents and formulations that were tested in the current study showed remarkable antifungal activity after being leached from PEM/THFM in a previous study (Salim et al., 2012a). These earlier findings highlight the importance of testing the degree of conversion and colour change to investigate the serviceability and durability of the suggested delivery system. However, these agents should not be routinely included into lining materials as this potentially forms a risk for the development of antimicrobial resistance as well as allergies for both acrylics (more exposure to monomers) and antimicrobials. Two formulations of fluconazole were used in this study, while in previous studies the formulations used were not clearly defined. Fluconazole capsules are commonly prescribed antifungal treatment and readily available for dental practice (Rautemaa and Ramage, 2011) and thus deserve to be investigated and compared to pure formulations. Further in vivo studies are warranted to investigate the usability and efficiency of this drug delivery system to treat oral candidal infections.
9.6 Conclusions

DC and colour stability of PEM/THFM systems were influenced by the addition of chlorhexidine and fluconazole. However, the values are comparable to other commonly used acrylic liners and within acceptable ranges. Therefore, despite the changes being statistically significant, their impact may not be of clinical relevance. PEM/THFM can be considered as a biocompatible delivery system. The advantages of incorporating these antifungal agents into denture linings for the treatment of an infection are likely to outweigh any adverse effects manifested by a minor reduction in the tested properties.
CHAPTER 10

General discussion, Conclusions and Suggestions for future research
10.1 General discussion

The prevalence and the aetiology of denture-induced candidosis were discussed extensively in the literature review (Chapter 1). Briefly, denture-induced candidosis is a common disease in elderly denture wearers and is a multifactorial disease and *C. albicans* is the principal causative agent. Other *Candida* species such as *C. glabrata* are commonly identified especially in medically compromised patients (Figueiral *et al.*, 2007; Coco *et al.*, 2008; Dagistan *et al.*, 2009; Rautemaa and Ramage, 2011). The management of this condition is complex due to its multifactorial aetiology. Predisposing factors should be addressed to prevent the recurrence of fungal infections. Moreover, denture-induced candidosis is a mixed multi-species biofilm infection which provides further challenges for its management and effective treatment requires disruption of this biofilm (Ramage *et al.*, 2005; Rautemaa and Ramage, 2011). Biofilm lifestyle is commonly associated with poor drug penetration and antimicrobial recalcitrance and microorganisms in biofilm are generally unaffected by host immune mechanisms (Cowen *et al.*, 2002; Ramage *et al.*, 2005).

Various protocols have been suggested for the treatment of patients suffering from oral candidosis (Samaranayake *et al.*, 2009; Rautemaa and Ramage, 2011). Although denture-induced candidosis could be treated by methods targeted towards the oral mucosa, other treatment modalities are directed toward the denture base. Cracks within the denture represent a yeast reservoir, which are difficult to clean by mechanical brushing (Ramage *et al.*, 2004). For successful treatment it is essential to eradicate the yeast from both the oral tissues as well as from the denture base (Kulak *et al.*, 1994; Ramage *et al.*, 2004).
Many therapeutic modalities are available ranging from denture disinfection to systemic antifungal therapy (Webb et al., 1998c; Uludamar et al., 2011). Despite this the recurrence rate of denture-induced candidosis is high (Lal et al., 1992; Cross et al., 2004). It has been suggested that this is due to poor access of the antifungals onto the fitting surface, their poor penetration into the microbial biofilm on the porous denture material, as well as their rapid clearance by saliva and tongue movements (Addy and Fugit, 1989; Cross et al., 2004).

Local drug carriers have been suggested to prolong the efficiency of oral treatment in order to maintain ideal therapeutic drug levels at the site of infection over the required period by release of the drug at a predetermined controlled rate (Douglas, 1977; Brook and van Noort, 1984; Geerts et al., 2008). Drug carriers are also convenient for patients as they do not require compliance to frequent application regimes. In addition, direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug-drug interactions. Favourable results for incorporation of antifungal agents in different polymeric systems have been reported (Addy, 1981; Patel et al., 2001; Geerts et al., 2008; Amin et al., 2009).

The aims of this research were to investigate the utility of poly(ethylmethacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) as a delivery system for sustained release of antimicrobials and to investigate the bioactivity of the leached antimicrobials against planktonic and biofilm candidal lifestyles. A further aim was to assess the impact of impregnation of a PEM/THFM polymeric delivery system with antimicrobials on a range of clinically important physical and mechanical properties of the acrylic liner.
Different methodologies have been employed in this research to verify the quantity of the leached antimicrobials (quantitative assessment) and to test the microbiological efficacy of the leached antimicrobials (qualitative assessment). In addition, different methodologies were used to test key mechanical and physical properties of the impregnated PEM/THFM acrylic system. The impregnated antimicrobials were chlorhexidine (CHX) and two formulations of fluconazole: pure (FLUp) and capsules (FLUc). The formulations of fluconazole used in previous studies were not clearly defined therefore both formulations were included in this study. Fluconazole capsules are readily available to dental practitioners, cheaper and are the commonly prescribed form.

Fluconazole is a systemic drug commonly used for the treatment of oral candidosis (Rautemaa and Ramage, 2011). However, in elderly patients with reduced saliva production, therapeutic levels form a risk for emergence of microbiological and clinical resistance (Siikala et al., 2010). Topical antifungals are effective but require daily compliance for frequent dosing (Geerts et al., 2008). Chlorhexidine is a widely used disinfectant with a remarkable antifungal, antibacterial and anti-biofilm potency (Ramage et al., 2011). Significantly, emergence of resistance has not been observed with chlorhexidine (Figueiral et al., 2007; Dagistan et al., 2009), but despite its high adsorption capacity such that it can be retained in the oral cavity for long periods, the maximum retention lasts for up to 12 h (Ellepola and Samaranayake, 2001). Impregnation of these agents into acrylic liners may prolong their efficiency.

The spectrum of the in vitro antifungal activity of CHX powder was evaluated first (Chapter 4). The antifungal activity of CHX against a panel of isolates belonging to a number of different Candida species commonly isolated from patients with oral
candidosis was tested, and compared against fluconazole activity. This was achieved by determining the minimum inhibitory concentration (MIC).

The findings showed that CHX has excellent broad-spectrum antifungal activity. It was effective at concentrations detected in saliva when using standard dosing regimens (Figure 4.2). Moreover, no cross-resistance was detected between chlorhexidine and fluconazole, also among Candida species highly resistant to fluconazole (Figure 4.1).

Based on the promising findings of the first experiment, a drug delivery system using PEM/THFM impregnated with CHX, FLUp or FLUc was established. The choice of a PEM/THEM polymeric system was based on its superior drug release characteristic compared to methyl methacrylate and n-butyl methacrylate based systems (Patel et al., 1994). This polymeric system enjoys excellent biological properties and is reported to be a ductile material (Pearson et al., 1986; Wyre and Downes, 2000).

The leached concentrations were investigated for 28 days (Chapter 5 and Chapter 6). Two methods were used to quantify the leached concentrations, namely bioassay and spectrophotometry and the methods were well correlated $R=0.99$. A total of 53.0 % of CHX, 38.5 % of FLUc and 13.2 % of FLUp impregnated into the discs was leached during the 28-day incubation detected by bioassay. Spectrophotometry detected 48.7 % of CHX, 40.79 % of FLUc and 14.7 % of FLUp. Of the total amount leached for all the impregnated antifungals 75 % was released during the first week of incubation. For all drugs a high rate of initial leaching was followed by a decreased but controlled sustained release during the entire test period of 28 days without reaching a plateau.
The initial high release is a surface phenomenon where the molecules at the surface are released at this early stage. The later slower diffusion is likely to be due to the diffusion of the drug from the core of the polymer by water cluster formation around the drug particles controlled by concentration dependent diffusion (Patel et al., 2001; Darwish et al., 2011) (Figure 10.1). The differences between the leached concentrations of different groups could be due to the difference in the solubility of the impregnated drugs. While pure fluconazole has a limited solubility (5 mg/ml at 37 °C), chlorhexidine is characterised by higher water solubility (19 mg/ml at 37 °C). Moreover, the molecular weight for chlorhexidine is twice that of fluconazole (Figure 8.4) which may also explain differences in their impact on water absorption; as the chlorhexidine particles leach out of the polymer they will leave larger voids behind absorbing more water compared to the voids created as a result of fluconazole leaching (Figure 10.1).

Importantly, the amount of drugs released exceeded the MIC values of most tested isolates even at the fourth week of incubation (Figure 5.2). This finding illustrates their wide spectrum antifungal activity. The significant difference in the leached concentration of fluconazole from the discs impregnated with FLUp and FLUc is likely to be due to the presence of impurities in the powder from capsules and the need to replace a higher proportion of the PEM powder in order to incorporate an equivalent amount of active drug into the discs. This may disrupt the polymer structure and allow the drug to leach more easily (Figure 10.1)
The bioassay method has been demonstrated to be highly sensitive and reproducible both in the quantitative and qualitative results (Chapter 5). Spectrophotometry was easy to use to quantify drug concentrations in the leachates (Chapter 6). However, bioassay has an advantage over spectrophotometry, as it can be used to verify the antifungal activity of the leached agents where inhibition zones can be detected.

The bioavailability and activity of the released drugs were investigated using four methods, namely: bioassay, time-kill study, XTT reduction assay, and crystal violet assay. The first two methods were used to assess the activity of the leached drugs against candidal planktonic lifestyle; however the latter two were used to assess the activity of the leached drugs against candidal biofilm lifestyle.

Figure 10.1 Schematic diagram showing the leaching mechanism over time in the polymeric delivery system impregnated with pure fluconazole (a), fluconazole from capsules (b), chlorhexidine (c)
Dental prostheses offer a perfect environment for candidal biofilm formation (Cuéllar-Cruz et al., 2012). This biofilm is predominantly produced by *C. albicans* as it has exceptional ability to attach to artificial surfaces (Rautemaa and Ramage, 2011; Cuéllar-Cruz et al., 2012). These *Candida* biofilms represent a significant aetiologial factor in denture-induced candidosis and its high relapse (Ramage et al., 2004). Therefore, management of candidal biofilms should be considered as a vital element in the treatment of oral candidosis. For these reasons, the efficiency of the suggested polymeric delivery system in this study has been tested against both planktonic and biofilm candidal lifestyles. In this study the antimicrobials were incorporated in the acrylic itself to be inherently available in the surface which is the target for biofilm formation. These impregnated acrylic surfaces were used as a substrate for *Candida* biofilm formation.

Using bioassay, clear inhibition zones of candidal growth for the leached concentrations were detected up to 28 days compared to the drug-free control (Chapter 5). Time-kill assay is a widely used analytical microbiological method for evaluation of antimicrobial activity of an antimicrobial agent against selected microorganism. The advantages of this test over all other microbiological tests are that the rate and the extent of microbial killing can be estimated (Kiraz et al., 2011). This assay was used (Chapter 6) to show precisely how quickly the leached agents were acting against the tested isolates which was a limitation for previously used methods such as CFU counts and well diffusion tests (Patel et al., 2001; Darwish et al., 2011). Using this assay CHX exhibited rapid killing and high fungicidal activity against all tested isolates, including the fluconazole-resistant isolates, compared to the two fluconazole formulations. The rate of activity of an antimicrobial is an important factor to consider and has a critical impact on the clinical outcome where rapid activity is
most favourable (Lewis et al., 2002). Three Candida isolates were tested: two C. albicans isolates and one C. glabrata were selected to assess the antifungal efficiency of the delivery system because they represent the most commonly associated species with denture-induced candidosis (Coco et al., 2008).

The superior fungicidal activity of CHX can be explained by the high leached concentrations of CHX which exceeded the MIC values (6.25 mg/L) for all isolates at all time intervals. Hiom et al (1995) demonstrated rapid pattern of uptake of CHX by C. albicans and C. glabrata within 30 s of exposure. Their results are in accordance with the results of the present study, as the antifungal activity of CHX against all tested isolates was within ≤60 s.

FLUc and FLUp were effective against the susceptible C. albicans isolate but showed markedly reduced activity against the resistant C. albicans isolate. Surprisingly, FLUp has demonstrated faster activity compared to FLUc, although FLUp was leached in lower amounts compared to FLUc. For example, FLUp showed more rapid decrease in viable counts of susceptible C. albicans within 60 min compared to 24 h for FLUc. In addition, the killing of C. glabrata was slower than that of the fluconazole-resistant C. albicans which is in agreement with previous reports (Li et al., 2003). These unexpected differences between the two formulations may be due to the presence of excipients in FLUc which may interfere with drug uptake by Candida and modify the action of the drug. The slow killing has significant consequences as it may lead to selection and emergence of resistance (Rautema and Ramage, 2011).

XTT reduction assay and crystal violet assay were used to quantify biofilm formation on the impregnated acrylic discs. These methods are widely accepted and are the most commonly used techniques for biofilm quantification (Pitts et al., 2003; Peeters et al.,
These methods quantify the biofilm formation differently: XTT measures the metabolic activity within biofilms while crystal violet measures the biomass of the biofilm.

The results of the biofilm study (Chapter 7) showed that CHX impregnated PEM/THFM acrylic material has remarkable anti-candidal biofilm activity, which is demonstrated by its ability to prevent biofilm biomass growth and biofilm metabolism up to 28 days. In contrast, FLUp impregnated discs inhibited biofilm formation on the discs poorly, with a maximum of 40% reduction in biofilm metabolic activity at two days. After this its activity was only slightly better than that of the drug-free control. This could be due to the very high sessile MIC which has been reported to be up to $4000 \times$ that of planktonic C. albicans cells for fluconazole (Ramage et al., 2004). This innate resistance to fluconazole has been explained by different mechanisms such as upregulation of efflux pumps and increased cell density (Ramage et al., 2005).

It has been shown that CHX reduces the adherence of C. albicans to surfaces (Jones et al., 1997; Pizzo et al., 2001), suppresses the hyphal formation (Kimura and Pearsall, 1980; Hazen et al., 1991; Ellepola and Samaranayake, 2000b) and significantly reduces cell surface hydrophobicity (Anil et al., 2001). The exceptional efficiency of CHX impregnated discs reported in this study is in agreement with previous studies that showed up to 100% reduction in the activity of pre-formed biofilm using CHX (Redding et al., 2009; Ramage et al., 2011).

Altering the chemical characteristics of biomaterials offers an effective solution for enhanced microbial control. Impregnation of dental polymers with antimicrobials is one of these methods. However, producing a suitable antimicrobial-containing
polymer presents a challenge of introducing antimicrobial properties while preserving the physical and mechanical properties of the polymer. The last two studies in this research were dedicated to investigate the impact of impregnation of the PEM/THFM polymer with CHX or FLU on the shear bond strength, water absorption, degree of conversion and colour stability of the material.

The impregnated polymer showed increased water absorption characteristics compared to the drug-free polymer. However, despite the increased water uptake, SBS remained acceptable for a temporary lining material and comparable to other drug-free long-term lining materials. In addition, even the lowest values seen with FLUc impregnated discs exceeded that of long-term lining materials (>0.7 MPa) (Hatamleh and Watts, 2008). Moreover, the enhanced water uptake could contribute to improved leaching which is a privilege for drug delivery system.

The DC and colour stability were influenced by the addition of chlorhexidine and fluconazole. However, the final values were comparable to other commonly used acrylic liners and within acceptable ranges. All investigated groups apart from the FLUc impregnated group fulfilled ISO specification 20795-1 criteria, where the residual monomer content should not exceed 4.5 % (International Organization for Standardization, 2008). PEM/THFM can thus be considered as a biocompatible drug delivery system.

The impregnated groups showed less favourable results compared to the drug-free control for all tested physico-mechanical properties. This could be related to the presence of the antimicrobials which may disrupt the polymer structure and introduce more spaces and less homogeneity in the resin matrix. Moreover, the agents may interfere with the polymerisation, precluding further propagation of the
chain (Riggs et al., 2000). The FLUc impregnated group was the least favourable compared to the other impregnated groups (Figure 10.1). This is likely to be due to the presence of impurities in the powder from capsules and the need to replace a higher proportion of the PEM powder in order to incorporate an equivalent amount of active drug into the discs. Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole), whereby inclusion at a level of 25 % is equivalent to inclusion of 10 % of pure fluconazole, as confirmed by bioassay and MIC testing (Appendix II). This may disrupt the polymer matrix and produce more porous structure resulting in weaker mechanical and physical properties (Figure 10.1).
10.2 Meanings and Implications

The clinical implications of this study relate to the clear need for new antifungal treatment modalities for denture-induced candidosis. The recurrence rate of this infection is high and persistent fungal presence on the fitting surface of dentures often leads to cross infection. In addition, the number of medically compromised patients, who are at high risk of infections, is dramatically increasing. Moreover, in patients with reduced saliva production it is difficult to achieve therapeutic drug levels in the oral cavity using systemic antifungal agents such as fluconazole. These facts highlight the importance of developing effective antifungal therapeutic treatment. Establishing an effective drug delivery system indicates the feasibility of this treatment modality for oral candidal infections, especially denture-induced stomatitis and difficult to treat infections with high relapse.

The economic implications are evident in that effective microbial control service will result in fewer clinical interventions and negate the need for repeated antifungal courses or replacement of failed interventions. Also, effective treatment of resistant strains and biofilms may lead to reducing the need for more complicated antifungal treatment, thus affording savings in time and resources. In addition, it is more convenient for the patient as it is a self-release system and it does not require compliance to frequent application regimes. Health service providers, policy makers, surgeons and eventually patients, will all benefit from improved microbial control and clinical performance.

The released amounts of all impregnated antimicrobial agents tested in this study are less than the maximum recommended doses. The average surface area for the acrylic discs used in this study was 2576 mm$^2$ and the average estimated surface area for
upper edentulous jaw is 4654±407 mm² in males, and 4212±368 mm² in females (Poštić, 2011). Therefore, the surface area for fitting denture is 1.6-1.8 times that of the surface area of acrylic discs used in the study. To simplify the calculations 1.8 (the upper limit) to be considered:

\[ \text{Average surface area of fitting denture} = 1.8 \times \text{Surface area of the acrylic disc} \]

Presuming that the release increases linearly with the increase of the surface area, we can expect the acrylic system on a denture to release 1.8× the amount of the impregnated drugs from the discs maximally. During the first day the released amounts were the highest (20.37 mg for CHX, 16.68 mg for FLU from FLUc discs, 3.92 mg for FLU from FLUp discs). Using the above presumptions a full denture would release 36.66 mg of CHX, 30.02 mg and 7.05 mg of FLU using FLUc and FLUp respectively. These amounts are within or even less than the allowed recommended doses (Rushton, 1977; Force and Nahata, 1995). Figure 10.2 illustrates the calculated amounts to be released for all impregnated drugs from a denture with average surface area.

The choice of the antifungal agent depends mainly on the antimicrobial profile of the causative agent, chronicity of the predisposing factors, immunological status of the patient and the presence of biofilms. Chlorhexidine could be used effectively when long-term treatment is needed and when biofilms are suspected. On the other hand, fluconazole can be successfully used to treat uncomplicated infections in otherwise healthy patients where risk for development of resistance is limited. The PEM/THFM may be used as a reline to existing dentures or as a base for new dentures for treatment of chronic or recurrent denture-induced candidosis. The suggested drug
delivery device ascertains a sustained release of the drugs throughout the treatment period.

Figure 10.2 The calculated amount (mg) of pure fluconazole (FLUp), fluconazole from capsules (FLUc) or chlorhexidine (CHX) to be released from denture with average surface area during 28-day treatment period. The straight lines represent the normal recommended dosing regimens, twice chlorhexidine rinsing daily (=40 mg) and single oral dose of fluconazole (=100 mg)
10.3 Strengths and Limitations

This investigation was conducted in a systematic approach to establish a new treatment modality of a clinically important problem. The modified standard microbiological investigations were carefully planned and validated for accuracy and reproducibility. In addition, all clinically safe and relevant drug concentrations were used. The current study has provided insights and bases that may inform further clinical studies.

The efficiency of the drug delivery polymeric system was investigated against both the planktonic as well as the biofilm candidal lifestyle. This is highly important given that the majority of human microbial infections involve biofilm formation. And it does imitate the lifestyle of microorganisms in the oral environment. In addition, it is inherently resistant to the most commonly prescribed antifungals. The choice of Candida species took into account not only susceptible and C. albicans species, but also covered a wide range of resistant isolates and different non-albicans Candida species.

The serviceability and durability of the suggested delivery system were investigated to verify its clinical performance. This part of the research was only conducted after promising results were obtained and all antifungal drugs and formulations that were tested showed excellent antifungal activity against a planktonic lifestyle after being leached from PEM/THFM. These findings reinforced the importance of testing the mechanical and physical properties of the impregnated polymer.

The main limitation of the present in vitro studies is that further in vivo work is warranted to determine their clinical relevance. However, a thorough spectrum of
aspects was looked at enabling rapid moving into clinical trials. The extensive study of \textit{in vitro} properties is the very first necessary step towards clinical application on patients. Also, a limited range of antimicrobial agents were used, however, the ones tested are the most commonly used and well tolerated agents. Moreover, distilled water was used as the incubation medium for the impregnated acrylic discs rather than saliva. This was done in order to improve the reproducibility of the model system. A previous study has shown higher released concentrations of the impregnated antifungals in artificial saliva compared to water (Darwish \textit{et al.}, 2011). Artificial saliva could have been a clinically more relevant but as an artificial solution may also be a source of artefact hence why water was used in these studies.

The concentrations of chlorhexidine in the leachates were 38 to 379 times higher than the mean MIC of the \textit{Candida} isolates. Although these concentrations are highly inhibitory, it is likely that lower concentrations with lower risk for side effects could also be efficient.

The number of isolates tested in the biofilm model and time-kill assay was small due to the laborious nature of these methods. A single species biofilm model may not reflect the efficacy against mixed multi-species biofilms. However, standard methods and reference strain for \textit{Candida} biofilm assays were used (Seneviratne \textit{et al.}, 2008; Ramage \textit{et al.}, 2011). The reference \textit{C. albicans} strain used in this study is a well known biofilm former and the most commonly used \textit{Candida} in biofilm studies. Moreover, CHX has shown good biofilm efficacy \textit{in vitro} and \textit{in vivo} against a broad-spectrum of oral microbes and is known to have good efficacy against biofilms (Thurmond \textit{et al.}, 1991; Ramage \textit{et al.}, 2011).
There are many more mechanical and physical properties than the ones considered in this research. However, the properties chosen to be tested are the most relevant to the oral cavity and covered a range of different physico-mechanical properties. The temperature, acidity, salts, and chemicals of food may have an impact on the mechanical and physical properties. However, these factors have limited access and hence effect on the fitting surface of a well-fitting denture and their impact is negligible especially for temporary-term use.


10.4 Conclusions

I. Based on the microbiological investigations and within the limitations of these studies the following conclusions can be drawn:

- Chlorhexidine exhibits excellent antifungal efficacy *in vitro* against a broad-spectrum of *Candida* species including fluconazole-resistant species.
- PEM/THFM polymeric system has potential as an effective carrier for sustained release of antifungal agents up to 28 days.
- The bioassay method demonstrates excellent sensitivity and reproducibility to quantify released drug concentrations in leachates and to verify their microbiological efficiency.
- Chlorhexidine leachates show rapid fungicidal activity against all *Candida* isolates tested and more rapidly than the two fluconazole formulation leachates.
- Chlorhexidine impregnated PEM/THFM acrylic shows excellent anti-candidal biofilm activity up to 28 days.
- Fluconazole impregnated PEM/THFM acrylic poorly inhibits biofilm formation on acrylic discs.

II. Based on testing the mechanical and physical properties of the impregnated polymeric system and within the limitations of these tests the following conclusions can be drawn:

- Incorporation of antimicrobials into PEM/THFM system significantly affects its physical and mechanical properties. However, final values are within acceptable ranges.
• Water storage has an impact on bond strengths: this negative impact did not affect the bond serviceability and durability.

• Impregnated PEM/THFM acrylic systems apart from the FLUc impregnated PEM/THFM are biocompatible based on their degree of conversion.

• Discs impregnated with FLUc are the least favourable in terms of mechanical and physical properties.

• The advantages of incorporating these antimicrobial agents into denture linings for the treatment of an infection are likely to outweigh any adverse effects manifested by a minor reduction in the tested properties.
10.5 Suggestions for future research

The feasibility of establishing a drug delivery system based on PEM/THFM was reported in this study. Although this work is an advance on many other previous studies by virtue of exploring the microbiological efficiency of the suggested model using different microbiological investigations and in relevance to clinical situations, the range of antimicrobials tested was not broad. Many antifungals and antimicrobials are currently being marketed such as echinocandins and tea tree oil: these agents are highly effective and further work investigating the efficiency of impregnating these agents is required.

There is a clear clinical need for evaluating the impact of this treatment modality on candidal gene expression and metabolism. It would be important to evaluate the impact of this on the mechanisms involved in fluconazole resistance and to further explain its poor efficiency against candidal biofilm lifestyle.

Further *in vitro* studies are required to determine the cytotoxic profile of the leached concentrations of chlorhexidine from PEM/THFM drug delivery system.

Based on the results of the current study a model for an *in vivo* study can be formulated. A clinical trial is essential in order to test the efficacy of the suggested drug delivery system for the treatment of denture-induced candidosis. An outlined protocol is provided in Appendix I for *in vivo* study.
REFERENCES


References


References


APPENDICES

APPENDIX - I  Protocol for in vivo study

Section 1- Patient selection and grouping

Patients of positively diagnosed denture-induced candidosis lesions are to be considered. The diagnosis should be confirmed clinically and using oral swap as a sampling technique. A parallel-group study design is to be used.

The exclusion criteria to be applied are:

1-allergy to acrylic denture base.
2-nutritional and hormonal deficiencies.
3-administration of oral antibiotics less than six months prior to the investigation.

Using the parallel-group study design, the candidate patients are to be divided into 5 groups as follows:

Group (1) comprised of patients whose dentures are to be relined with drug-free PEM/THFM acrylic "control"

Group (2) comprised of patients who are to be treated with topical antifungal gel (such as: Nystatin oral gel)

Group (3) comprised of patients who are to be given an antifungal tablets “Fluconazole 100 mg/day”

Group (4) comprised of patients whose dentures are to be relined with drug delivery acrylic system containing 10 % Chlorhexidine.

Group (5) comprised of patients whose dentures are to be relined with drug delivery acrylic system containing 10 % Fluconazole.
Section 2-Clinical procedures

- Upper alginate impressions are to be taken for each patient before treatment (baseline) and at regular weekly intervals during treatment for up to one month.
Section 3-Microbiological investigations

- The impressions are to be used as swaps which are to be cultured in Saborauds agar ‘impression culture’, incubated for 72 h at 37 °C. This is to be followed by counting the Colony Forming Units “CFU” of Candida albicans for each patient at each interval. The collected data will be recorded and the first CFU count will be used as a control for that corresponding patient.
APPENDIX - II  MIC values of FLUp against MIC values of FLUc for different *Candida* species

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<tr>
<td>SA</td>
<td><em>C. kefyr</em></td>
<td>0.5 mg/L</td>
<td>0.5 mg/L</td>
</tr>
</tbody>
</table>
Fungicidal amounts of antifungals are released from impregnated denture lining material for up to 28 days

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ABSTRACT
Objectives: The aim of this study was to investigate the efficacy of a polymeric delivery system impregnated with chlorhexidine or flucanazole against Candida species.

Methods: Self-cure poly-ethyl methacrylate and tetrahydro-furfuryl methacrylate (PEM/TM) discs impregnated with pure flucanazole substance (FLUP), flucanazole powder from capsules (FLUC) or chlorhexidine powder (CHX) were incubated in water for up to 28 days at 37 °C. The water was replaced at 24 h and 3, 7, 14, 21, 28 days. The amount of released drug and antifungal activity of the leachates was measured by bioassay. The minimal inhibitory concentration (MIC) of each drug for 46 Candida isolates was determined and compared to the released concentrations.

Results: A total of 53.0% of CHX, 38.5% of FLUC and 13.2% of FLUP impregnated into the discs was leached during the 28-day incubation. Of the total amount leached, 71.8% of CHX, 75.1% of FLUC and 70.5% of FLUP was released during the first week of incubation. Antifungal activity was confirmed for up to 28 days.

Conclusion: Both chlorhexidine and flucanazole become readily leached from PEM/TM polymer up to four weeks and that the polymerisation of the acrylic does not affect the antimicrobial activity of the agents. Importantly, the amount of drugs released exceeded the MICs of most isolates also during the fourth week of incubation.

Clinical significance: These findings indicate the feasibility of this treatment modality for oral candidal infections, especially denture stomatitis. Further in vivo work is warranted to determine its clinical relevance and applicability.

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1. Introduction

Candida-associated denture stomatitis is a common disease in elderly denture wearers with a prevalence of 45–70%.\textsuperscript{3,4} It is a multifactorial disease and Candida albicans is the principal causative agent. Other Candida species such as \textit{C. glabrata} are commonly identified especially in medically compromised patients.\textsuperscript{3,4} Many therapeutic modalities are available ranging from denture disinfection to systemic antifungal therapy.\textsuperscript{5,6} Despite this the recurrence rate of denture stomatitis is considerably high.\textsuperscript{7,8} It has been suggested that this is due to
poor access of the antifungals onto the fitting surface, their poor penetration into the microbial biofilm on the porous denture material, as well as their rapid clearance by saliva and tongue movements. A drug commonly used for the treatment of oral candidiasis is systemic fluconazole. However, in elderly patients with reduced saliva production, therapeutic levels form a risk for emergence of microbiological and clinical resistance. Topical antifungals are effective but require daily compliance for frequent dosing. Chlorhexidine is a widely used disinfectant with a remarkable antifungal, antibacterial and anti-biofilm potency. Significantly, emergence of resistance has not previously been observed with chlorhexidine.

Local drug carriers have been suggested to prolong the efficiency of oral treatment as ideal therapeutic drug levels can be maintained at the site of infection over the required period by release of the drug at a predetermined controlled rate. Drug carriers are also convenient for patients as they do not require compliance to frequent application regimes. In addition, direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug-drug interactions. Favourable results with incorporation of different antifungal agents in polymeric systems used in dentures have been reported. Chow et al. have shown that denture liner containing nystatin, fluconazole or itraconazole has detectable antifungal activity. It has also been demonstrated that mixing chlorhexidine, fluconazole, nystatin or clotrimazole into a soft lining material results in inhibition of candidal growth, demonstrating the release of the drugs from the liner. Ryu et al. demonstrated that chlorhexidine could be released steadily from self-cured poly (methyl methacrylate) acrylic throughout the 28-day test period. Trujillo et al. incorporated nystatin with two different soft liners and demonstrated a constant fungicidal activity up to 14 days. A self-cured poly (ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PFM/THF) polymeric system has been shown to have good biocompatibility, unique water uptake properties and potential for a drug carrier. A dose-dependent release pattern of chlorhexidine impregnated into PFM/THF has been demonstrated with excellent inhibition of candidal growth. Moreover, exceptional water uptake characteristics of PFM/THF acrylic resin is considered an important attribute to enhance drug delivery of the acrylic system.

Various techniques have been used to measure the release of the antifungal drugs from the drug carriers. Spectrophotometry is easy to use and provides reliable results of drug concentrations in solutions, whereas HPLC is technically more demanding but is more accurate. However, neither of these methods measures the bioavailability and activity of the released drug. Bioassay is a well known technique used widely in microbiological investigations. It is commonly employed to measure the drug concentrations in plasma samples from patients and provides both quantitative and qualitative information about the released drugs.

The aim of the present study was to investigate the utility of PFM/THF as a delivery system for a sustained release of antifungals using a bioassay technique. This system has previously been used as a drug carrier for chlorhexidine but its applicability as a carrier for fluconazole has not been successfully tested. Furthermore, the bioactivity of the leached drugs has not been previously analysed using a qualitative and quantitative method such as bioassay. The hypothesis was that PFM/THF system could be used for the effective local delivery of fluconazole and chlorhexidine in the treatment of denture stomatitis.

2. Materials and methods

2.1. Study design

Five parallel poly(ethyl methacrylate)/tetrahydrofurfuryl methacrylate (PFM/THF) discs were prepared by replacing a proportion of the PFM by pure fluconazole (FLU) or fluconazole powder from capsules (FLUc) or chlorhexidine (CHX). Five control discs were prepared following manufacturer’s instructions. All discs were incubated in sterile distilled water for up to 28 days at 37°C. The water was collected and replaced at 24 h and 3, 7, 14, 21, 28 days. The amount of released drugs and antifungal activity of the leachates was measured by bioassay. The minimal inhibitory concentration (MIC) of each drug for a total of 46 Candida isolates was determined in the bioassay.

2.2. Preparation of PFM/THF Discs

For the control discs, 1 g of PFM (Lucite, Durham, UK) and 0.6 ml of THF (Sigma-Aldrich, Dorset, UK) were mixed. For the impregnated discs, 10% (w/w) of the PFM was replaced with CHX (Sigma-Aldrich), 10% (w/w) with FLU (Sigma-Aldrich) or 25% (w/w) with FLUc (Pfizer, Kent, UK). Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25% of it is equivalent to 10% of pure fluconazole, as confirmed by bioassay and MIC testing (data not shown). The discs were blended into the PFM, then poured into the THF liquid monomer and mixed. All mixtures were packed into disc shaped steel moulds (80 mm diameter and 0.5 mm height) and allowed to cure for 15 min.

2.3. Disc incubation

Discs were soaked individually in 20 ml of sterile distilled water at 37°C in tightly sealed 100 ml sterile plastic containers. The containers were gently shaken 3 times daily throughout the experiment to simulate the clinical situation. Drug concentrations within the leachates were detected using a bioassay method. The leachates of the control discs (acrylic without drug) were used as negative controls.

2.4. Bioassay

Bioassay was performed following a standard protocol. A suspension of Candida albicans San Antonio strain or C. albicans (ATCC90028) was mixed into melted agar together with Yeast Nitrogen Base with Glucose (1%) and Tris-sodium citrate (0.05%) (YNBG + citrate) solution and poured into a plate (245 mm × 245 mm). Wells of 8 mm diameter were cut out in the agar. Two-fold dilution series of pure fluconazole and
chlorhexidine were prepared and these known concentrations were used as drug standards and internal controls. Triplicates of drug standards (FLU and CHX), duplicates of internal controls (FLUc and CHX) and duplicates of the leachate test specimens were dispensed in the wells. Furthermore, duplicates of THFM and PEM diluted into dimethyl sulfoxide (DMSO) were tested. After overnight incubation at 37°C, zones of inhibition around each well were measured using digital callipers (Mitutoyo, Hampshire, UK). The mean inhibition zones of the drug standards were plotted as a regression curve, which was used as a standard graph. This was used to calculate the drug concentrations in the test samples and in the internal controls. To pass the quality control requirement the internal controls had to be within 20% of the expected concentration of each drug.

The bioassay was validated for linearity, precision, accuracy and reproducibility. The limit of detection was calculated as the smallest consistently detectable zone of inhibition (Lower Limit Of Quantification: LLOQ) and an overall coefficient of variation (CV) less than 20% was accepted. Standard curves were judged linear if the coefficient of determination (r) using linear regression was >0.99. Acceptance criteria dictated that the mean result must be within 20% of the theoretical concentration of LLOQ, and within 15% for all other samples. The reproducibility of the method was assessed by re-analysing 30% of the samples of each group on different days and applying Bland and Altman statistical analysis.

2.5. Susceptibility testing against chlorhexidine and fluconazole

Susceptibility testing against chlorhexidine and fluconazole was performed on a total of 46 Candida isolates comprising of 32 C. albicans, 13 C. glabrata and one C. krusei isolate. MICs were determined using FLUp and CHX and CLSI M27-A3 microdilution methodology. Briefly, two-fold dilution series of fluconazole (0.125–2048 mg/L) and chlorhexidine (0.1–50 mg/L) prepared in sterile distilled water and an inoculum of 1 x 10^7 organisms/mL was used. After 48 h incubation at 37°C, the growth in each well was measured by spectrophotometry (at 490 nm). For fluconazole, the minimum inhibitory concentration (MIC) was the lowest drug concentration that reduced the OD_{590} by 50% compared to the drug-free control. The CLSI standard breakpoints for fluconazole were used for susceptibility interpretation. For chlorhexidine, the MIC was the lowest drug concentration that prevented any discernible growth (80%). Geometric means (GM) and ranges were calculated.

2.6. Statistical analyses

Normality was established allowing a parametric one-way ANOVA (SPSS) to be used to analyse all data and the significance level was set at P ≤ 0.001. All results were tested using Leven's test for homogeneity of variance (P ≤ 0.05), following the assumption of equal variances. Equal variances were confirmed (P > 0.05), hence the Bonferroni post hoc test was used to determine the differences in the leached amounts of the impregnated drugs at each time interval.

Table 1 - The cumulative amount (mean ± SD; in mg) of pure fluconazole (FLUp), fluconazole from capsules (FLUc) or chlorhexidine (CHX) released into the distilled water from the acrylic discs during 28-day incubation measured at 6 different time points. Differences between groups were statistically significant at all time points (P < 0.001).

<table>
<thead>
<tr>
<th>Days</th>
<th>Amount released (mean ± SD), in mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHX</td>
</tr>
<tr>
<td>1</td>
<td>21.4 (1.5)</td>
</tr>
<tr>
<td>1-3</td>
<td>30.8 (0.1)</td>
</tr>
<tr>
<td>5-7</td>
<td>38.1 (2.2)</td>
</tr>
<tr>
<td>7-14</td>
<td>44.4 (2.2)</td>
</tr>
<tr>
<td>14-21</td>
<td>49.2 (0.1)</td>
</tr>
<tr>
<td>21-28</td>
<td>53.0 (0.1)</td>
</tr>
</tbody>
</table>

3. Results

A total of 53.0% of CHX, 38.5% of FLUc and 12.3% of FLUp impregnated into the PEM/THFM discs was leached during the 28-day incubation as detected by bioassay (Table 1). For all drugs a high rate of initial leaching was followed by a decreased but controlled sustained release during the entire test period of 28 days. Of the total amount leached, 71.8% of CHX, 75.1% of FLUc and 70.5% of FLUp was released during the first week of incubation (Fig. 1). During the fourth week of incubation 190 mg/L of CHX, 120 mg/L of FLUc and 50 mg/L FLUp was released from the discs (Fig. 2). The release rate of CHX was significantly higher compared to FLUc and FLUp at all time intervals (P < 0.001). In addition, a significantly higher amount of fluconazole was leached from the FLUp discs than from the FLUc discs at all time intervals (P < 0.001). All internal controls were within 10% of the expected values.

Antifungal activity was confirmed for CHX, FLUc and FLUp impregnated disc leachates by clear inhibition zones of candidal growth compared to the known drug standards in the bioassay (Fig. 3). The leachates of the negative control discs (acrylic without drug) or the two components of the polymer

![Fig. 1 - Proportion of drug leached from discs impregnated with chlorhexidine (CHX), fluconazole from capsules (FLUc) and pure fluconazole (FLUp) during the 28-day incubation. Cumulative percentage presented.](image)
Fig. 2 – MICs (mg/L) of 46 isolates (left y-axis) and the amount drugs released from discs impregnated with fluconazole powder from capsules (a), pure fluconazole (b) and chlorhexidine (c) during the 28-day incubation (right y-axis).

alone (THFM and PEM) showed no inhibition of candidal growth. Bland and Altman analyses for the repeated measurements (9 in each group) showed minimal variation between results and high reproducibility (Fig. 4). The assay was linear with $r^2 > 0.99$ and the limits of detection were 6.25 and 50 mg/L for fluconazole and chlorhexidine, respectively. Accuracies were within 7% for fluconazole and 8% for chlorhexidine, and variability (precision) was <3% for both fluconazole and chlorhexidine.

The MIC values of fluconazole and chlorhexidine against 46 isolates of Candida are shown in Fig. 2. The GM MIC of all isolates was 1.83 mg/L (0.25–256) for fluconazole and 4.77 mg/L (0.78–6.25) for chlorhexidine. The GM MIC of the C. albicans isolates for fluconazole was 0.61 mg/L (0.25–256) and for chlorhexidine 5.03 mg/L (1.56–6.25) while the GM MIC of the C. glabrata isolates for fluconazole was 30.34 mg/L (8–128) and for chlorhexidine 4.78 mg/L (3.25–625). The MIC for the one C. kefyr isolate for fluconazole was 0.5 mg/L and for chlorhexidine was 0.78 mg/L.

Fig. 3 – A photograph of a representative bioassay plate showing growth inhibition zones formed by diluted leachates of chlorhexidine (a) and fluconazole (THFM) (b) discs in distilled water. A random template was used for well selection. Positive controls in 1A wells, negative controls, e.g. in 2C (b) and 1D (b), THFM controls in 4E (b) and 3B (b) and PEM controls in 6B (b) and 6E (b).
Fig. 4 – The reproducibility of the bioassay was assessed by re-analysing 30% of the samples of each group (CHX, FLUc, FLUp). A Bland and Altman plot of 27 repeated measurements (9 each group) shows minimal variation between results and high reproducibility. Each dot represents the mean concentration of two measurements against the difference between the two measurements. 95% Confidence intervals were 4.93–10.84 for the first measurements and 4.69–10.24 for the second measurements.

4. Discussion

The present study shows that it is feasible to employ the PEM/THFM polymeric system for sustained release of antifungal agents up to 28 days and accordingly our hypothesis has been accepted. Furthermore, our results clearly indicate that both chlorhexidine and fluconazole are readily leached from the polymer and polymerisation of the acrylic does not affect the antimicrobial activity of the incorporated agents even in prolonged incubation. Importantly, the amount of drug released from the discs each week exceeded the MICs of most isolates. The amount of chlorhexidine released from the discs during the last week of incubation exceeded the MICs of all isolates, and the amount of fluconazole released exceeded the MICs of 97.8% of the isolates during the second week of incubation and 91.3% of the isolates during the last week of incubation for FLUc and FLUp, respectively. Following the initial high release, the elution of drugs showed a slow and steady diffusion without reaching a plateau after 28 days. The slower diffusion is likely to be attributed to the diffusion of the drug from the core of the polymer by water cluster formation around the drug particles controlled by concentration dependent diffusion. The polymeric system used in this study is characterised by distinctive water absorbing capability and sustained drug release.

The amount of chlorhexidine released was significantly higher than that of the two fluconazole preparations. This is in agreement with previous findings using a different polymer carrier. However, the amount of chlorhexidine released in our study was markedly higher than previously reported for PEM/THFM carrier using spectrophotometry. This may be due to the differences in the dimensions and surface areas of the discs. A proportionally larger surface area exposes more drug particles to the soaking water and thus enhances drug release. Moreover, spectrophotometry is generally regarded to be less sensitive than bioassay as a detection method, although there is no single study to confirm this. Significant differences were detected in the leaching of fluconazole from the discs impregnated with pure fluconazole and fluconazole powder from capsules. This is likely to be due to the presence of impurities in the powder from capsules and the need to replace a higher proportion of the PEM powder in order to incorporate equivalent amount of active drug into the discs. This may disrupt the polymer structure and allow the drug to leach more easily. The formulation of fluconazole used in previous studies was not clearly defined whereby both formulations were included in our study. Fluconazole capsules are readily available to dental practitioners and based on the results of this study could be useful in clinical practice.

Highly inhibitory concentrations of chlorhexidine and fluconazole released from the PEM/THFM discs. The concentrations of fluconazole released from FLUc discs ranged from 1380 to 197 times higher than GM MIC for C. albicans, 47 to 4 times for C. glabrata, and 2390 to 240 times for C. kefyr during the first and last weeks, respectively. One C. albicans and three C. glabrata isolates with high MIC values for fluconazole and designated as resistant were included in order to test the efficacy of the PEM/THFM on difficult to treat organisms. The concentrations of fluconazole released from discs impregnated with FLUc were higher than the MICs of the resistant C. glabrata isolates up to 21 days of incubation but only up to 3 days of leaching of FLUp discs. The MIC for the resistant C. albicans was higher than the amount of fluconazole released from FLUc discs after 3 days of incubation and at all times of that of pure fluconazole. The released concentrations of chlorhexidine ranged from 379 to 38 times higher than the GM MIC for C. albicans, 399 to 40 times than the GM MIC for C. glabrata and 2424 to 243 times than the GM MIC for C. kefyr during the first and last week, respectively. Non-cross-resistance between fluconazole and chlorhexidine was detected and the leached concentrations of chlorhexidine were higher than the MICs of all isolates at all time points. The components of the polymer system showed no antimicrobial effect.

Direct comparison of our results with previously reported studies is difficult due to differences in the methodologies, drug concentrations, formulations and detection methods used. However, the results of the present study are in line with those of Byalat et al. showing similar release and efficacy of chlorhexidine from PEM as they did from PMMA. Truhlar et al. have demonstrated a similar decrease of antifungal activity during extended water immersion but in their study the released concentrations were not compared with MICs of candidal isolates as in the present study. The main limitation of the present and the above-mentioned in vitro studies is that further in vitro work is warranted to determine their clinical relevance. Nevertheless, the quantitative and qualitative bioassay method used in the present study could be a valuable tool for evaluation of other polymeric systems and other antifungal agents both in vitro and in vivo. Whilst the bioassay method demonstrated good sensitivity and reproducibility.
In conclusion, the PEM/THF polymeric system was found to be an effective carrier for sustained release of antifungal agents up to 28 days. However, the polymeric system impregnated with CHX or FLU showed remarkable antifungal activity against a wide range of Candida isolates and the released concentrations were within the non-toxic range. The choice of the antifungal agent depends mainly on the antimicrobial profile of the causative agent, chronicity of the predisposing factors, immunological status of the patient and the presence of biofilms. Chlorhexidine could be used effectively when an ankle has failed, in immunocompromised patients with a need for long-term treatment, as well as when mixed bacterial and fungal biofilms are suspected. On the other hand, fluconazole is well tolerated with few adverse effects and can be successfully used to treat uncomplicated infections in otherwise healthy patients where risk for development of resistance is limited. Therefore, a polymeric system containing CHX or FLU could be a very promising treatment modality as the drug is effective and directed to the site of pathology.

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REFERENCES


APPENDIX - IV  Publication 2

Impregnation with antimicrobials challenge bonding properties and water sorption behaviour of an acrylic liner

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ABSTRACT

Objectives: To investigate the effect of impregnation of poly (ethyl methacrylate) and tetrahydrofuranyl methacrylate (PDM/TMFM) polymeric delivery system with chlorhexidine or fluconazole on its shear bond strength (SBS) and water sorption.

Methods: For SBS testing, 16 PDM/TMFM discs impregnated with chlorhexidine (CHX), pure fluconazole (FLU) or fluconazole from capsules (FLUC) and 16 drug-free control discs were prepared and bonded to heat-cured acrylic blocks. All discs were allowed to set for 24 h at room temperature.

After setting, the half discs (n = 8) were tested immediately (Group 1). The other half was further incubated in water for 28 days at 37 °C before testing (Group 2). To evaluate water uptake, five PDM/TMFM discs impregnated with CHX, FLU or FLUC and five drug-free control discs were prepared and incubated in water. Mass changes were measured up to six months.

Results: The mean SBS for control, FLU and FLUC were 4.01, 3.85, 3.29 and 2.26 MPa, respectively for Group 1. Group 2 showed significantly lower SBS (P ≤ 0.05). All failures were adhesive.

The percentage mass change due to water sorption ranged significantly from 12% for control to 27% for FLUC (P ≤ 0.05). A strong negative correlation between the extent of water absorption and the SBS was detected (R = 0.94, P = 0.05).

Significance: Impregnation with antimicrobials presents a challenge to the physical and mechanical properties of a polymer. However, despite increased water uptake SBS remained acceptable for a temporary lining material and comparable to drug-free long-term lining materials. Moreover, the enhanced water uptake could contribute to improved leaching.

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1. Introduction

A wide range of drug delivery systems has been suggested to be used for the treatment of many oral conditions by incorporation of antimicrobial agents with denture acrylic resin or with soft liners. Using polymerised acrylic as a carrier for drugs orally is a method to extend the duration of effective therapy. The use of drug carriers is convenient for patients as they do not require compliance for frequent application regimes. In addition, direct delivery of the drug to the site of infection reduces the risk of systemic side effects or drug-drug interactions. Incorporation of different antifungal agents in polymeric systems used in dentures has shown remarkable efficiency.

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Various polymeric systems have been suggested for the controlled release of bioactive agents in the oral cavity. Many of these systems are based on poly(methylmethacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) complex materials, which have superior characteristics compared with methyl methacrylate and n-butyl methacrylate based systems, where it has low exothermic reaction compared to the former and superior biological properties compared to the latter. This system was reported to be a ductile material, which is a good attribute for prostheses. Moreover, the same system was documented to enjoy excellent biological properties such as supporting growth and viability of chondrocytes and being non-irritant in dental use. Mechanical properties such as flexural strength, hardness, and strength bonding to the underlying substrate are vital characteristics for intraoral liners and prostheses. A strong bond between the medicated lining and underlying acrylic denture is also critical for safe durable and effective delivery of the drug for the required treatment period. However, the incorporation of drugs into the acrylic may compromise its mechanical and physical properties. A marked reduction in compressive and tensile strength has been demonstrated in bone containing more than 8% of antibiotics. Equally, soft linings impregnated with nystatin showed a noticeable increase in water uptake. Many studies have investigated the bond strength of liners bonded to denture base resin using different tests such as tensile, shear or peel tests. However, less is known about the mechanical and physical properties of the PEM/THFM polymeric system, and the impact of incorporation of antimicrobial agents on its different properties has not been evaluated.

In view of the limited research in this area, this study was set out to assess whether incorporation of antimicrobial agents compromises the clinical performance and the properties of the PEM/THFM acrylic material. The aims of this study were (i) to investigate the shear bond strength of PEM/THFM containing chlorhexidine and fluconazole to heat cured acrylic substrate in standard conditions as well as after storage in distilled water and (ii) to investigate the water uptake of PEM/THFM containing chlorhexidine and fluconazole. The null hypotheses were that (1) the shear bond strength would not be significantly affected by impregnating the PEM/THFM acrylic system with antimicrobial agents and (2) the water uptake behaviour of the drug-free PEM/THFM discs is not significantly different compared to the impregnated discs.

### Materials and methods

#### Study design

The impact of incorporation of chlorhexidine (CHX), pure fluconazole (FLUp) or fluconazole powder from capsules (FLUc) on shear bond strength (SBS) and water sorption of poly(methyl methacrylate)/tetrahydrofurfuryl methacrylate (PEM/THFM) was tested in vitro. For SBS, 16 parallel PEM/THFM discs were prepared by replacing a proportion of the PEM by CHX, FLUc or FLUc (described in detail below). Sixteen control discs were prepared following manufacturer’s instructions (Table 1). All discs at dough stage were bonded to heat-cured acrylic blocks and were allowed to cure for 24 h (the time for complete setting). Then the discs were allocated into two different groups (n = 8). Group 1 discs were tested immediately and in Group 2 discs were further stored in water for 28 days at 37°C. All specimens were tested using shear bond strength test. For water sorption analyses, a total of 20 discs were prepared. Of these, 15 PEM/THFM discs were prepared by replacing a proportion of the PEM by CHX, FLUc or FLUc. Five control discs were prepared following manufacturer’s instructions. All discs were incubated in water at 37°C and mass changes were measured for up to 6 months.

#### Shear bond strength

For SBS testing, a heat cure PMMA acrylic resin (Skilbond Ltd., Bucks, UK) was prepared following manufacturer’s instructions. Powder and liquid were mixed in a 3:1 ratio until plastic dough was obtained. After mixing the material was packed inside a hollow cylindrical brass mould (external diameter = 18 mm, internal diameter = 16 mm, depth = 25 mm). A 6 h long cycle was applied using a 95°C hot water bath (the first 4 h at 60°C). The surfaces were prepared for bonding 24 h after fabrication by rubbing with 60 grit silicone carbide waterproof abrasive paper. The acrylic surfaces were wiped with acetone and left to dry. Subsequently, a thin and homogenous layer of an adhesive bond primer (Principality Medical Ltd., Newport, UK) was applied with a brush onto the surface, and the surfaces were left to dry for 30 min at room temperature according to the manufacturer’s instructions.

To prepare the drug-free control discs (n = 16), a cold cure-polymerised acrylic resin (PEM/THFM) was prepared using ratio of 1 g of PEM powder (Lucite, Durham, UK) to 0.6 ml of THFM liquid (Sigma-Aldrich, Dorset, UK). Materials used in this study are presented in Table 1. Three medicated groups (n = 16) were prepared by replacing a proportion of the PEM by CHX, FLUc or FLUc.

### Table 1 – Groups of the shear bond study: control, chlorhexidine (CHX), fluconazole pure (FLU) and fluconazole from capsules (FLUc).

<table>
<thead>
<tr>
<th>Groups (n = 16)</th>
<th>Acrylic substrate</th>
<th>Powder/liquid ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>3:1 heat cured PMMA</td>
<td>1.0:6 PEM/THFM</td>
</tr>
<tr>
<td>CHX group</td>
<td>3:1 heat cured PMMA</td>
<td>1.0:6 PEM/THFM with 10% by weight of CHX substituted in powder</td>
</tr>
<tr>
<td>FLUc group</td>
<td>3:1 heat cured PMMA</td>
<td>1.0:6 PEM/THFM with 10% by weight of FLUc substituted in powder</td>
</tr>
<tr>
<td>FLUc group</td>
<td>3:1 heat cured PMMA</td>
<td>1.0:6 PEM/THFM with 25% by weight of FLUc substituted in powder</td>
</tr>
</tbody>
</table>
were prepared \((n = 16)\). The drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed: 10% (w/w) of the PEM was replaced with CHX (Sigma–Aldrich) 10% (w/w) by FLUP (Sigma–Aldrich) or 25% (w/w) by FLUC (Pfizer, Kent, UK) (resulting in 100 mg of active drug per disc; for all). Powder from fluconazole capsules contains a high amount of excipients (150 mg of excipients per 100 mg of fluconazole) whereby 25% of it is equivalent to 10% of pure fluconazole, as confirmed by bioassay and minimum inhibitory concentration testing (MIC) (data not shown). The PEM/THFM mixtures of all groups were packed against the primed surface in the dough stage using teflon discs (external diameter = 18 mm, internal diameter = 8 mm, thickness = 3 mm) to produce a constant area of bonding for all samples (Fig. 1).

After 24 h curing of all samples, samples were allocated into two different main groups \((n = 8)\): Group 1 discs were tested immediately and in Group 2 discs were further stored in water for 28 days at 37 °C. For water storage, samples were separately immersed in 100 mL distilled water and left in a thermostatically controlled cabinet at 37 °C for 28 days. All samples were debonded using a shear test jig installed on a Zwick/Roell 2020 testing machine using a 500 N load cell (Zwick/Roell, Leominster, UK). The shear jig complies with ISO/TR 11405:1994 standard and 20 kN loading cell was used. Cross head speed was set at 1 mm/min. The force-to-failure was recorded \(N\) and the type of bond failure was determined and classified as adhesive, cohesive or combined failure (Fig. 1). Examination of the specimens for adhesive or cohesive failures was carried out visually and judged by two examiners. The shear bond strength (SBS) was calculated according to the following equation:

\[
\text{SBS} = \frac{F}{A} \quad (1)
\]

where \(F\) stands for maximum force to failure expressed in \(N\), \(A\) is the area of attachment expressed in \(\text{mm}^2\) and SBS represents the shear bond strength (MPa). Data are presented as mean of SBS and standard deviations.

### 2.3. Water sorption

For water sorption investigation, the drug-free control discs were prepared by mixing 1 g of PEM and 0.6 mL of THFM. For the impregnated discs, the drugs were blended into the PEM, then poured into the THFM liquid monomer and mixed as described above for SBS. All mixtures were packed into disc shaped steel moulds (40 mm diameter and 0.5 mm height) and allowed to cure for 15 min.

All discs \((n = 5)\) were stored in desiccators with anhydrous silica gel at 37 °C and weighed to \(\pm 0.01\) mg daily using a calibrated electronic analytical balance with a precision of 0.01 mg (Ohaus Analytical Plus, Ohaus Corporation, USA), until the mass change of each disc was constant and not more than 0.1 mg in any 24 h period this was to ensure complete dehydration. This constant mass is the baseline mass \((m_0)\) for the discs. The discs were then soaked individually in 100 mL of sterile distilled water at 37 °C in tightly sealed sterile plastic containers. Readings were taken daily for the first week and then weekly up to two months and then every month up to 6 months. For each measurement samples were carefully removed from the water with tweezers, dried on filter paper until free from visible moisture, air-dried for 15 s and weighed and returned to the water bath. The recorded mass at any given time was denoted as \((m_t)\). The percentage apparent mass change \((M)\) was calculated by:

\[
M = \frac{m_2 - m_1}{m_1} \times 100
\]

The data is presented as mean percentage of mass change.

### 2.4. Statistical analysis

The bond strength results and water sorption for all groups were analysed by one-way ANOVA and Dunnett T3 post hoc test at 0.05 significance level. Linear regression was used to test the correlation between water uptake measured in this study and amount drug leached reported previously.

### 3. Results

Mean shear bond strengths for all groups are shown in Table 2. For Group 1 (tested immediately), the control discs of cured
PfM/THFM resin revealed the highest SBS (4.01 MPa) which was significantly higher compared to SBS of FLUc impregnated discs (P < 0.001) and CHX impregnated discs (P = 0.046), but not for FLUp (P = 1.00). The difference was not statistically significant between FLUp discs (3.85 MPa) and CHX discs (3.29 MPa) (P = 0.47). After water incubation for 28 days (Group 2), the control discs of cured PfM/THFM showed the highest SBS (2.89 MPa) with a significant difference between all stored specimens (P < 0.001). The SBS was significantly lower in all water-stored specimens (Group 2) compared to their corresponding Group 1 specimens (P < 0.05, for all) as shown in Fig. 2. The mode of failure was adhesive at the lining/base interface in all specimens (Fig. 1).

Fig. 3 shows the mean percentage of mass change against square root of time (t^1/2) of PfM/THFM with the presence and absence of the antifungal agents after immersion in water for up to 6 months. A significant increase in mass after water storage was detected compared to the initial mass for all discs (P < 0.05). The control absorbed 12% water compared to 19%, 23% and 27% for FLUp, CHX and FLUc impregnated discs respectively after 6 months. The mass change was significantly different for the control discs when compared to FLUp, CHX and FLUc (P = 0.004, P = 0.001, P ≤ 0.001 respectively) after 6 months. After 28 days of water absorption the mass change between all groups was significantly different (P < 0.001). PfM/THFM did not equilibrate after 6 months.

The correlation between mass change and square root of time in the early stages of water uptake was linear reflecting a diffusion-controlled uptake. A strong negative correlation between the water absorption and SBS was demonstrated (R = 0.94, P = 0.05). In addition, a significant positive correlation between water uptake measured in this study and amount drug leached reported previously was detected for all drugs: (R = 0.99, P < 0.001) for CHX (R = 0.876, P = 0.022) for FLUp and (R = 0.817, P = 0.047) for FLUc.3

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**Table 2 – Mean (SD) of shear bond strength (MPa) for all discs (Group 1 tested immediately) and Group 2 water-stored discs (water immersion for 28 days). These groups consisting of subgroups of control discs without impregnation, discs impregnated with pure fluconazole (FLUp), chlorhexidine (CHX), and fluconazole from capsules (FLUc) (different upper case alphabets in rows indicate significant difference at P ≤ 0.05).**

<table>
<thead>
<tr>
<th>Tested groups</th>
<th>Sub-groups</th>
<th>Control</th>
<th>FLUp</th>
<th>CHX</th>
<th>FLUc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td>4.01 (0.47)</td>
<td>3.85 (0.49)</td>
<td>3.29 (0.45)</td>
<td>2.26 (0.35)</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td>2.89 (0.33)</td>
<td>2.11 (0.10)</td>
<td>1.45 (0.15)</td>
<td>1.12 (0.18)</td>
</tr>
</tbody>
</table>

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**Fig. 2 – A bar chart showing the mean shear bond strength (standard deviation) under two different conditions: (1) the specimens stored dry at room temperature (Group 1) to be tested after 24 h, and it comprises 4 sub-groups: control discs, discs impregnated with chlorhexidine (CHX), pure fluconazole (FLUp) or fluconazole from capsules (FLUc). (2) The specimens were further immersed in water at 37 °C for 28 days after 24 h setting (Group 2) and it consists of the same sub-groups of discs. Horizontal capped lines indicate significant difference between the dry stored discs and their corresponding water stored discs (P ≤ 0.05).**

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**Fig. 3 – A graph of percentage mass change (%) versus time (min^1/2) of control discs, discs impregnated with chlorhexidine (CHX), fluconazole from capsules (FLUc) and pure fluconazole (FLUp) immersed in distilled water for 6 months (510.5 min^1/2).**
4. Discussion

It has been previously shown by the authors of the current study that microbiologically efficient levels are leached from PEM/THFM polymeric system impregnated with fluconazole or chlorhexidine. The results of the present study show that the impregnation of PEM/THFM with chlorhexidine or two formulations of fluconazole has a significant impact on their water uptake and bond strength to heat cured acrylic substrate. Consequently, both null hypotheses were rejected. However, the detected bond strength is to be considered sufficient and durable to service (−0.44 MPa). In addition, even the lowest values seen with FLUC impregnated discs exceeded that of long term lining materials (−0.7 MPa). There are no previous reports exploring the SBS and the water uptake of the PEM/THFM system when impregnated with antimicrobials. Importantly, the impact of the three incorporated agents was found to be different. Fluconazole from capsules showed a more adverse impact on SBS and water uptake compared to both fluconazole pure and chlorhexidine. The polymeric system used in this study showed a remarkable water absorbing capability compared to other similar polymeric systems. This could be beneficial for a drug delivery system and may result in enhanced drug release. However, the possibility of leaching residual monomer increases as the water absorption increases. Nevertheless, this polymeric system is suggested to be used for a temporary use with well documented biologically satisfactory characteristics. Interestingly, a significant positive correlation between the water uptake measured in this study and the amount of drug leached reported previously was detected for all drugs. However, water uptake does not necessarily reflect the leached drug levels if the compound contains impurities and excipients like FLUC.

The increased water uptake in the impregnated discs could be due to the formation of droplets around the impregnated drug particles which will enlarge as the water is diffusing in due to the osmotic gradient between the internal droplet and the external solution. Another factor that may play a role in the water uptake is the solubility of the impregnated drug. While pure fluconazole has a limited solubility (5 mg/ml at 37 °C), chlorhexidine is characterised by higher water solubility (19 mg/ml at 37 °C) compared to fluconazole. Moreover, the molecular weight for CHX is twice as much as the molecular weight of FLU (Fig. 4) which may also explain differences in their impact on water absorption; as the CHX particles leach out of the polymer they will leave larger voids behind absorbing more water compared to the voids created as a result of fluconazole leaching.

Bond strengths of PEM/THFM to heat cured PMMA were influenced by impregnating the former with antimicrobial agents under standard and conditioned circumstances. This could be related to the presence of the antimicrobials which may disrupt the polymer structure and introduce more spaces and less homogeneity in the resin matrix and thus potentially weaken the bond strength between the doped lining and the denture. Moreover, the agents may interfere with the polymerisation, precluding further propagation of the chain. All bond strength failures were adhesive failures. This indicates that the bond strength between the lining and the acrylic is weaker than the lining strength which is an advantage for a temporary lining in practice. The further reduction in the bond strength after immersion in water can be explained by the distinctive water absorption capability of the PEM/THFM polymer. This finding is reflected by the strong negative correlation between the bond strength and the water absorption capability. This finding is in line with other studies that tested different resilient liners.

Significant differences were detected between the influence of pure fluconazole and fluconazole powder from capsules. This is likely to be due to the presence of excipients in the powder from capsules and the need to replace a higher proportion of the PEM powder in order to incorporate equivalent amount of active drug into the discs. Powder from fluconazole capsules contains a high amount of excipients.
(150 mg of excipients per 100 mg of fluconazole) whereby 25% of it is equivalent to 10% of pure fluconazole, as confirmed by bioassay and MIC testing (data not shown). This may disrupt the polymer structure and produce more porous structure which reflects weaker bond strength and higher sorption ability. The formulations of fluconazole used in previous studies were not clearly defined therefore both formulations were included in our study. Fluconazole capsules are readily available to dental practitioners and are the commonly prescribed form which worth to be investigated in this study.

The storage period for SBS was decided based on the average time suggested in the literature for the drug delivery device usage. The samples were incubated in distilled water at 37 °C to simulate the clinical situation. The water absorption from biological fluids such as saliva is much lower than that in distilled water as a result of the increased osmolarity which limits the water uptake. Consequently the bond strength can be expected to be higher in the oral cavity than that tested in distilled water in vitro. The shear bond test used in the present study does not represent the sum of acting forces in the oral cavity. However, it relates well to the loads that affect the lining materials during function and it does offer useful information on how a modification of the lining material may affect its mechanical properties. Importantly, all antifungal drugs and formulations that were tested in the current study showed excellent antifungal activity after being leached from PEM/THFM in our previous study. These findings reinforce the importance of testing the bond strength and water absorption characteristics to investigate the serviceability and durability of the suggested delivery system in clinical use. Further studies are warranted to investigate the usability and efficiency of this drug delivery system in vivo.

5. Conclusions

Within the limitations of this work, the following conclusions may be drawn:

1. Incorporating antifungal agents into PEM/THFM resin by polymer substitution significantly affects its bond strength to acrylic substrate and the water uptake characteristics.
2. Immersing samples in water has a significant influence on the bond strength as a result of distinctive water absorption ability of the PEM/THFM.
3. Despite the reduction in the bond strengths after water storage, all bonds are still serviceable and clinically durable to be used as a long term lining for effective therapy.
4. The mode of failure was adhesive in all specimens.

REFERENCES


